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(54) Title: ATTENUATED RESPIRATORY SYNCYTIAL VIRUS

(57) Abstract

Attenuated respiratory syncytial viruses (RSV) and in particular temperature sensitive RSV are provided. The viruses of the present invention may be used in pharmaceutical compositions such as vaccines. Methods of making and using such pharmaceutical compositions are also provided.

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ATTENUATED RESPIRATORY SYNCYTIAL VIRUS

RELATED APPLICATIONS

The present application is a continuation-in-part of PCT International Application No. PCT/US97/05588 (designating the United States), filed April 3, 1997, which claims priority under 35 U.S.C. §119(e) from U.S. Serial No. 60/014,848 , filed April 4, 1996. Both applications are hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention relates generally to attenuated respiratory syncytial viruses and, more particularly, to live attenuated respiratory syncytial virus vaccines and methods of protecting against disease caused by infection with respiratory syncytial virus.

BACKGROUND OF THE INVENTION

Respiratory syncytial virus (RSV), a member of the paramyxoviridae family, is the leading cause of viral pneumonia and bronchitis in infants and young children worldwide, and is a major cause of fatal respiratory tract disease. Serious disease is most prevalent in infants 6 weeks to 6 months of age and in children with certain underlying illnesses (*e.g.,* immunodeficiencies, congenital heart disease and bronchopulmonary dysplasia). Virtually all children are infected by two years of age. Most infections are symptomatic and are generally confined to mild upper respiratory tract disease. A decrease in severity of disease is associated with two or more prior infections and, in some studies, with high levels of serum antibody, suggesting that protective immunity to RSV disease will accumulate following repeated infections (Lamprecht, C.L. et al., *J. Inf. Dis.* 134:211-217 (1976); Henderson, F.W. et al., *N. Eng. J. Med.* 300:530-534 (1979); Glezen, W.P. et al., *J. Ped.* 98:706-715 (1981); Glezen, W.P. et al., *Am. J. Dis. Child.* 140:543-546 (1986); Kasel, J.A. et al., *Vir. Immunol.* 1:199-205 (1987/88); Hall, C.B. et al., *J. Inf. Dis.* 163:693-698 (1991)).

Two major subgroups of RSV have been identified, A and B, as well as antigenic variants within each subgroup (Anderson, L.J. et al., *J. Inf. Dis.* 151:626-633 (1985); Mufson, M.A. et al., *J. Gen. Virol.* 66:2111-2124 (1985)). Multiple variants of each subgroup have been found to co-circulate in epidemics which occur annually during late fall, winter, and spring months (Anderson, L.J. et al., *J. Inf. Dis.* 163:687-692 (1991)). There is evidence that children infected with one of the two major RSV subgroups may be protected against reinfection by the homologous subgroup (Mufson, M.A. et al., *J. Clin. Microbiol.* 26:1595-1597 (1987)). This, along with evidence that protective immunity will accumulate following repeated infections,

suggests that it is feasible to develop an RSV vaccination regiment for infants and young children which would provide sufficient immunity to protect against disease and death.

A previous attempt to vaccinate young children against RSV employed a parenterally administered formalin-inactivated RSV vaccine. Unfortunately, administration of this vaccine in several field trials was shown to be associated with the development of a significantly exacerbated illness following subsequent natural infection with RSV (Kapikian, A.Z. et al., *Am. J. Epidemiol.* 89:405-421 (1968); Kim, H.W. et al., *Am. J. Epidemiol.* 89:422-434 (1969); Fulginiti, V.A. et al., *Am. J. Epidemiol.* 89:435-448 (1969); Chin, J. et al., *Am. J. Epidemiol.* 89:449-463 (1969)).

Following the lack of success with the formalin-inactivated RSV vaccine, emphasis was placed on the development of live attenuated vaccines. For example, cold adaptation, a process by which virus is adapted to grow at temperatures colder than those at which it normally optimally grows, has been used to develop temperature sensitive, attenuated RSV mutants for consideration as vaccines (Maassab, H.F. et al., *Vaccine* 3:355-369 (1985)). Unlike chemical mutagenesis in which the genetic lesions are usually single, this method generally results in the accumulation of multiple genetic lesions. These multiple lesions would help to confer phenotypic stability by reducing the probability that reversion of any one lesion will result in reversion to virulence. Stepwise cold adaptation, wherein the virus is passaged multiple times at progressively lower temperatures, has been used to successfully develop several influenza vaccine candidates currently in clinical trials (Maassab, H.F. et al., *Viral Vaccines* Wiley-Liss, Inc. (1990); Obrosova-Serova, N.P. et al., *Vaccine* 8:57-60 (1990); Edwards, K.M. et al., *J. Inf. Dis.* 163:740-745 (1991)). These mutants, which bear attenuating mutations in at least four different genes, appear to be attenuated, immunogenic, and phenotypically stable.

RSV was cold-adapted to 25-26°C in several laboratories in the mid-1960's, but was found to be under-attenuated in vaccine trials (Kim, H.W. et al., *Pediatrics* 48:745-755 (1971); Maassab, H.F. et al., *Vaccine* 3:355-369 (1985)). However, it is of note that administration of these live RSV vaccine candidates was never associated with disease enhancement following natural infection.

Live attenuated vaccines offer several advantages over inactivated vaccines. These include the possible use of a single dose and administration by the natural route of infection *i.e.*, intranasally. In addition, live attenuated vaccines stimulate a wide range of immune responses, including local and serum antibody responses and

cellular immunity. Furthermore, these vaccines are cost-effective and can be rapidly produced and updated in the event of antigenic changes.

It would thus be desirable to provide avirulent (attenuated), immunoprotective and genetically-stable live attenuated RSV strains. It would further be desirable to provide a vaccine comprising such attenuated strains. It would further be desirable to provide methods of making and using said RSV vaccine to protect against disease caused by infection with RSV.

SUMMARY OF THE INVENTION

Attenuated RSV strains which exhibit the cold-adapted (*ca*) and/or temperature sensitive (*ts*) phenotype are provided. Samples of viruses as embodiments of the present invention have been deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20582, under the terms of the Budapest Treaty, and have been accorded the following ATCC designation numbers:

Table 1

Virus	Phenotype	Description	ATCC Designation No.	Date of Deposit
CRla	temperature sensitive/cold-adapted	RSV, la-CRSV-5 CL 15 MRC27	VR-2511	September 20, 1995
Ca19S	temperature sensitive/cold-adapted	RSV, Line 19 MRC5-15-25° st-33°	VR-2512	September 20, 1995
19H	temperature sensitive	RSV, Line 19 MRC5-60-35°	VR-2513	September 20, 1995
Ca48V	temperature sensitive/cold-adapted	RSV, Line 48 MRC5-14-25° st MRC1-33° VERO 10-25° VERO 1-33°	VR-2514	September 20, 1995
Ca19V	temperature sensitive/cold-adapted	RSV, Line 19 MRC5-10-25° VERO 16-25° VERO 6-20° VERO 3-33°	VR-2515	September 20, 1995
CaBCV	temperature sensitive/cold-adapted	RSV, CRSV-BC5 CL-17 MRC30	VR-2516	September 20, 1995
CaBCL	temperature sensitive/cold-adapted	RSV, CRSV-BC13 MRC19-25° MRC1-33°	VR-2517	September 20, 1995
19H 4MD	temperature sensitive	RSV, Line 19 MRC5 92-35°C, Clone 4-1, MRC5, P-103-33° Purified by minimal limited dilution (MLD)	VR-2567	April 2, 1997
19H 3PI	temperature sensitive/cold-adapted	RSV, Line 19 MRC 70-35°C, Vero 3-35°C, MRC5 2-35°C Clone 2-35°C (3PI) Plaque purified	VR-2564	April 2, 1997

Table 1 (con't.)

Virus	Phenotype	Description	ATCC Designation No.	Date of Deposit
19H 5MD	temperature sensitive	RSV, Line 19 MRC5, 92-35°C Clone 5-1 Purified by MLD	VR-2565	April 2, 1997
CRla MD	temperature sensitive/cold-adapted	RSV, la-CRSV-5 MRC-38-25°C, MRC1-33°C Purified by MLD	VR-2566	April 2, 1997
19HL 3PI	temperature sensitive/cold-adapted	RSV, Line 19 MRC5 72-35°C VERO 3-35°C Large Clone 6-35°C, (3PI) Plaque Purified	VR-2572	April 23, 1997
wt 19		Progenitor wt RSV, line 19 MRC5 1-33°C, VERO 2-35°C, MRC5 1-35°C Purified by MLD	VR-2570	April 23, 1997
WRSV		Progenitor wt RSV, WI 38-3 MRC5 9-35°C, MRC5 1-35°C, Purified by MLD	VR-2571	April 23, 1997

The deposit of the viruses will be maintained in the ATCC depository, which is a public depository, for a period of 30 years, or 5 years after the most recent request, or for the effective life of a patent, whichever is longer, and will be replaced if the deposit becomes depleted or nonviable during that period. Samples of the deposited strains will become available to the public and all restrictions imposed on access to the deposits will be removed upon grant of a patent on this application.

The present invention also provides methods for immunizing a subject against disease caused by infection by RSV comprising administering to the subject an immunoeffective amount of an attenuated RSV and in particular, cold-adapted and/or temperature sensitive RSV. Methods of making and using such attenuated RSV in a pharmaceutical composition *e.g.*, a vaccine, are also provided.

Additional objects, advantages, and features of the present invention will become apparent from the following description, drawings and appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The various advantages of the present invention will become apparent to one skilled in the art by reading the following specification and subjoined claims and by referencing the following drawings in which:

Figure 1A is a graph showing the total anti-F IgG response of mice immunized with an immunogenic composition of an aspect of the present invention;

Figure 1B is a graph showing the anti-F IgG_1 response of mice immunized with an immunogenic composition of an aspect of the present invention;

Figure 1C is a graph showing the anti-F IgG_{2a} response of mice immunized with an immunogenic composition of an aspect of the present invention;

Figure 2A is a graph showing the anti-RSV-F antibody titers (after 4 weeks) of mice immunized with an immunogenic composition of an aspect of the present invention;

Figure 2B is a graph showing the anti-RSV-F antibody titers (after 8 weeks) of mice immunized with an immunogenic composition of an aspect of the present invention;

Figure 3 is a graph showing RSV specific neutralizing antibody titers (after 4 and 8 weeks) of mice immunized with an immunogenic composition of an aspect of the present invention;

Figure 4 is a graph showing cytotoxic T cell (CTL) activity of mice immunized with an immunogenic composition of an aspect of the present invention; and

Figure 5 is a graph showing CTL activity of mice immunized with immunogenic compositions of aspects of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

Attenuated RSV including cold-adapted and/or temperature sensitive RSV, as well as progenitor viruses, are provided and have been deposited with the ATCC and are described in detail herein. As used herein, the term "cold-adapted" means a virus that has been attenuated by propagation at lower than optimal growth temperatures and the term "temperature sensitive" means that replication of the virus is impeded as temperature is elevated.

The lines of the present invention have been successfully attenuated using three different approaches: adaption to suboptimal temperature by direct and stepwise passage; high passages at 35°C; and, adaption to a heterologous host (*i.e.*, host-range restricted). Four of the deposited lines have also been plaque purified (19HL 3PI; 19H 3PI, 19H MD and CRIa MD). The attenuated RSV of the present invention are genetically-stable, immunogenic and protective, and avirulent, and are thus particularly useful in the formulation of live, attenuated RSV vaccines which are capable of eliciting a protective immune response without causing unacceptable symptoms of severe respiratory disease. The immune response which is achieved in the subject by the method of an embodiment of the present invention preferably includes the production of virus specific neutralizing antibodies and the virus specific cytotoxic T-cell responses. The invention is therefore particularly effective to provide protection against respiratory tract diseases caused by RSV.

Methods of attenuating RSV, for example, attenuating the deposited progenitor viruses, as well as methods of making and using attenuated RSV vaccines, are also provided by the present invention, including the preparation of pharmaceutical compositions.

Nucleic acid molecules encoding the attenuated RSV are also within the scope of the present invention. These nucleic acids may be DNA molecules, cDNA molecules or RNA molecules *e.g.*, antisense RNA. The present invention further includes nucleic acid molecules which differ from that of the nucleic acid molecules which encode the RSV of the present invention, but which produce the same cold-adapted and temperature sensitive phenotypic effect. These altered, phenotypically equivalent nucleic acid molecules are referred to as "equivalent nucleic acids." The present invention also encompasses nucleic acid molecules characterized by changes

in non-coding regions that do not alter the phenotype of the polypeptide produced therefrom, when compared to the nucleic acid molecules of the RSV described herein.

Also encompassed by the present invention are the nucleic acid molecules comprising noncoding sequences of the RSV of the present invention. These non-coding regions are to include 5′ noncoding regions, 3′ noncoding regions, intergenic sequences, and other noncoding regions of the viral genome. These include, but are not limited to, transcriptional, translational and other regulatory regions. These nucleic acid molecules also may be DNA molecules, cDNA molecules or RNA molecules.

Nucleic acid molecules which hybridize under stringent conditions to the nucleic acid molecules described herein are also within the scope of the present invention. As will be appreciated by those skilled in the art, multiple factors are considered in determining the stringency of hybridization including species of nucleic acid, length of nucleic acid probe, T_m (melting temperature), temperature of hybridization and washes, salt concentration in the hybridization and wash buffers, aqueous or formamide hybridization buffer, and length of time for hybridization and for washes. An example of stringent conditions are DNA-DNA hybridization with a probe greater than 200 nucleotides in 5 x SSC, at 65°C in aqueous solution or 42°C in formamide, followed by washing with 0.1 x SSC, at 65°C in aqueous solution. (Other experimental conditions for controlling stringency are described in Maniatis, T. et al., *Molecular Cloning: a Laboratory Manual*, Cold Springs Harbor Laboratory, Cold Springs, N.Y. (1982) at pages 387-389 and Sambrook, J. et al., *Molecular Cloning: a Laboratory Manual*, Second Edition, Volume 2, Cold Springs Harbor Laboratory, Cold Springs, N.Y., at pages 8.46-8.47 (1989)).

The nucleic acid molecules of the present invention may be operatively-linked to a promoter of RNA transcription, as well as other regulatory sequences. As used herein, the term "operatively-linked" means positioned in such a manner that the promoter and other regulatory sequences will direct the transcription off of the nucleic acid molecule. An example of a promoter is the T7 promoter. Vectors which contain both a promoter and a cloning site to which an inserted piece of nucleic acid is operatively-linked to the promoter, are well known in the art. Preferably, these vectors are capable of transcribing nucleic acid *in vitro* and *in vivo*.

Purified polypeptides isolated from the RSV described herein or from cells infected with these same virus, are also encompassed by the present invention. The

polypeptides (or fragments thereof) may be of varying length, and preferably will be capable of exhibiting immunological activity.

Methods for producing polypeptides of the present invention are also within the scope of the present invention. In one embodiment, RSV polypeptides can be isolated in substantially pure form from RSV or cultures of cells infected with RSV. In an alternative embodiment, RSV polypeptides can be isolated from a recombinant system or are vector-engineered to produce these polypeptides. In yet another embodiment, RSV polypeptides can be chemically synthesized by methods well known to those of skill in the art.

All derived RSV strains including the deposited attenuated RSV derived from the progenitor viruses, are also encompassed by the present invention, including, without limitation, those attenuated by cold adaptation (including both direct and stepwise passage), high *in vitro* passage, host-range restriction and chemical or genetic modification *e.g.*, site-directed mutagenesis.

Although the deposited RSV of the present invention are subgroup A virus, it will be appreciated by those skilled in the art that subgroup B virus can be produced by biologically cloning wild-type subgroup B virus in an acceptable cell substrate using methods known in the art. The subgroup B virus may then be attenuated as described herein.

Pharmaceutical compositions comprising any of the RSV described herein or polypeptides, either alone or in combination, and a pharmaceutically acceptable carrier, are also provided by the present invention. As used herein, the phrase "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as physiologically balanced culture medium, phosphate buffered saline solution, water, and emulsions, such as an oil/water emulsion, various types of wetting agents and protein stabilizers. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Formulations include those suitable for oral, nasal, topical (including transdermal, buccal and sublingual), parenteral (including subcutaneous) and pulmonary administration. The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any method known in the art.

In one embodiment of the present invention, the pharmaceutical composition is intended for use as a vaccine. In such embodiment, a virus may be mixed with cryoprotective additives or stabilizers such as proteins (*e.g.*, albumin, gelatin), sugars

(*e.g.*, sucrose, lactose, sorbitol), amino acids (*e.g.*, sodium glutamate), saline, or other protective agents. This mixture may then be desiccated or lyophilized for transport and storage, and reconstituted prior to administration. Lyophilized virus will typically be maintained at about 4°C and when ready for use, reconstituted in a stabilizing solution, with or without adjuvant. In yet another embodiment of the present invention, the virus may be inactivated and may be mixed with an adjuvant, saline and a detergent such as phosphate Tween buffer. For further methods of vaccine preparation, see Duffy, J.I., *Vaccine Preparation Techniques*, Noyes Data Corporation, (1980).

Immunogenicity can be significantly improved if the virus is co-administered with an immunostimulatory agent or adjuvant. Adjuvants enhance immunogenicity but are not necessarily immunogenic themselves. Immunostimulatory agents or adjuvants have been used for many years to improve the host immune responses to, for example, vaccines.

Suitable adjuvants are well known to those skilled in the art and include, without limitation, aluminum phosphate, aluminum hydroxide, QS21, Quil A, derivatives and components thereof, calcium phosphate, calcium hydroxide, zinc hydroxide, a glycolipid analog, an octodecyl ester of an amino acid, a muramyl dipeptide, polyphosphazene, a lipoprotein, ISCOM matrix, DC-Chol, DDA, and other adjuvants and bacterial toxins, components and derivatives thereof.

Pharmaceutical compositions comprising any of the attenuated RSV of the present invention are useful to immunize a subject against disease caused by RSV infection. Thus, this invention further provides methods of immunizing a subject against disease caused by RSV infection, comprising administering to the subject an immunoeffective amount of a pharmaceutical composition of the invention. This subject may be an animal, for example a mammal, such as a primate or preferably a human.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, immunogenic and protective. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the immune system of the individual to synthesize antibodies, and, if needed, to produce a cell-mediated immune response. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and may be monitored on a patient-by-patient basis. However, suitable dosage ranges are readily determinable by one skilled in the art

and generally range from about 10^2 to about 10^9 plaque forming units (PFU) or more of virus per patient, more commonly, from about 10^4 to about 10^5 PFU of virus per patient. The dosage may also depend, without limitation, on the route of administration, the patient's state of health and weight, and the nature of the formulation.

It will be appreciated that administration of the vaccines of the present invention will be by procedures well established in the pharmaceutical arts, such as intranasally, parenterally, intravenously, orally, or topically applied to any mucosal surface such as intranasal, oral, eye or rectal surface. Moreover, as described in more detail in Specific Example 3E., more than one route of administration may be employed either simultaneously or sequentially (*e.g.*, boosting). In a preferred embodiment of the present invention, live, attenuated viral vaccines are administered intranasally, orally, parenterally or applied to any mucosal surface (nasal, oral, eye, rectal). Inactivated whole virus vaccine is preferably administered parenterally or to any mucosal surface.

Upon inoculation with an attenuated RSV pharmaceutical composition as described herein, the immune system of the host responds to the vaccine by producing antibodies, both secretory and serum, specific for RSV proteins. As a result of the vaccination, the host becomes at least partially or completely immune to RSV infection, or resistant to developing moderate or severe RSV infection, particularly of the lower respiratory tract.

It will be appreciated that the attenuated RSV of the present invention can be combined with viruses of other subgroups or strains to achieve protection against multiple strains of RSV. Typically the viruses will be in an admixture and administered simultaneously, but may also be administered separately. Due to the phenomenon of cross-protection among certain strains of RSV, immunization with one strain may protect against several different strains of the same or different subgroup.

In some instances it may be desirable to combine the attenuated RSV vaccines of the present invention with vaccines which induce protective responses to other agents, particularly other childhood viruses. For example, the vaccine compositions of the present invention can be administered simultaneously, separately or sequentially with other vaccines such as parainfluenza virus vaccine, as described in Clements, et al., *J. Clin. Microbiol.* 29:1175-1185 (1991). Moreover, a multivalent preparation may be employed comprising for example, the attenuated RSV of the

present invention (including subgroups A and B), parainfluenza virus type 1, 2 and 3 and influenza virus types A and B.

It will also be appreciated that single or multiple administrations of the vaccine compositions of the present invention may be carried out. In neonates and infants, multiple administration may be required to elicit sufficient levels of immunity. Administration should begin within the first month of life, and continue at intervals throughout childhood, such as at two months, six months, one year and two years, as necessary to maintain sufficient levels of protection against wild-type RSV disease. Similarly, adults who are particularly susceptible to repeated or serious RSV infection, such as, for example, health care workers, day care workers, elderly and individuals with compromised cardiopulmonary function, may require multiple immunizations to establish and/or maintain protective immune responses. Levels of induced immunity can be monitored by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to maintain desired levels of protection.

Those skilled in the art will further appreciate that the viruses of the present invention may be used in diagnostic applications. For example, a method of determining the presence of antibodies specifically reactive with an RSV of the present invention is provided. Such a method comprises the steps of contacting a sample with the RSV to produce complexes comprising the virus and any antibodies present in the sample specifically reactive therewith, and determining production of the complexes. A similar method of determining the presence of RSV is provided wherein the sample is contacted with an antibody specifically reactive with an RSV to produce complexes comprising the antibody and the virus present in the sample that is specifically reactive with the antibody, and determining production of the complexes.

The virus of the present invention are characterized by a level of attenuation such that they do not produce RSV disease in a host immunized therewith, evoke a protective immune response and do not lead to immunopotentiation or exacerbated disease. They lack transmissibility, are genetically stable and exhibit cold-adapted and temperature sensitive markers. They are immunogenically protective and induce protective levels of humoral and cell mediated immunity. In particular, a balanced anti-RSV F IgG1/IgG2a response is seen in hosts immunized with attenuated viruses of the present invention. They can be administered by the natural route *i.e.*, intranasally. The RSV of the present invention may be tested in *in vitro* and *in vivo*

models to demonstrate these characteristics. A variety of animal models have been described and are summarized in Meignier et al., eds., *Animal Models of Respiratory Syncytial Virus Infection*, Merieux Foundation Publication (1991). A cotton rat model of RSV infection is described in U.S. 4,800,078 and Prince et al., *Virus Res.* 3:193-206 (1985), and is believed to be predictive of attenuation and efficacy in humans. A primate model of RSV infection using a chimpanzee is also useful in examining attenuation and protection and is described in detail in Richardson et al., *J. Med. Virol.* 3:91-100 (1978) and Wright et al., *Infect. Immun.* 37:397-400 (1982).

SPECIFIC EXAMPLES

The following Specific Examples illustrate practice of the invention. These examples are for illustrative purposes only and are not intended in any way to limit the scope of the claimed invention.

Specific Example 1 describes the production and characterization of the virus of the present invention including the passage status and procedures for developing the strains.

Specific Example 2 describes temperature sensitivity studies, wherein the deposited strains were found to have the *ts* phenotype (see Tables 2 and 3).

Specific Example 3 describes immunogenicity studies wherein mice were immunized by administering an RSV strain of the present invention. As described in Specific Examples 3A.-3D., mice were immunized and sera examined four weeks after boosting for anti-F, total IgG antibodies, IgG1 and IgG2a antibodies. The results are shown in Figures 1A-3 and Tables 4-7 and show that the intranasal immunization with the attenuated RSV produces a substantial anti-F antibody response. In particular, a balanced anti-RSV F IgG1/IgG2a response demonstrating the induction of both Th-1 and Th-2 type responses was achieved. The generation of IgG2A antibodies in the murine model is indicative of a Th1-type immune response. The level of virus-neutralizing antibodies was also determined, by plaque reduction assays.

In Specific Example 3E., a study was performed to evaluate the effect of boosting by a route of administration that differs from the initial inoculation route of administration. In particular, mice were inoculated intranasally and boosted in the footpad and intramuscularly. Neutralization titer data is set forth in Table 8.

As described in Specific Examples 3F. and 3G., the generation of RSV-specific cytotoxic T cells (CTL) following immunization was determined and the results shown in Figures 4 and 5. Immunizing animals with the attenuated RSV of the present invention induced significant levels of CTL activity. In addition, as shown in Table 9,

mice immunized with the plaque purified viruses of the present invention were protected against live virus challenge.

Specific Example 4 describes sequence analysis of the F gene of several RSV lines. Specific Example 4A. sets forth the comparison of the sequences for wild type RSV (referred to herein as WRSV) and two attenuated lines, Ca19V and 19H (see Table 10). Specific Example 4B. illustrates the comparison of the sequences for the line 19 progenitor strain (referred to herein as wt 19) and the same two lines, lines Ca19V and 19H (see Table 11). The F genes of the two attenuated lines both differ from the WRSV as well as the wt 19, but have 66 nucleotides and 11 amino acids in common. With respect to the amino acid differences between the attenuated lines and the wt 19, none of the amino acid differences are shared by the two attenuated lines.

Specific Example 5 describes the plaque purification of the attenuated RSV lines of the present invention. Table 12 illustrates the temperature sensitivity of the plaque purified lines.

Specific Example 6 describes RSV therapeutic protocols for administering the pharmaceutical compositions of the present invention to humans.

SPECIFIC EXAMPLE 1 - PRODUCTION AND CHARACTERIZATION OF VIRUS

A. The following sets forth the deposited RSV strains and their titers.

1. 19H
   in EMEM 5% FBS 5% Glycerol
   1.00×10^8 $TCID_{50}$ in MRC Tubes on Day 14
   1.85×10^5 Pfu's/ml in VERO cells
   ATCC Designation No. VR-2513

2. Ca19S
   in EMEM 5% FBS 5% Glycerol
   7.00×10^4 Pfu's/ml in VERO cells
   ATCC Designation No. VR-2512

3. Ca19V
   in 199 5% FBS 5% Glycerol
   1.00×10^7 Pfu's/ml in VERO cells
   ATCC Designation No. VR-2515

4. Ca48V
in 199 5% FBS 5% Glycerol
9.00 x 10^4 Pfu's/ml in VERO cells
ATCC Designation No. VR-2514

5. CaBCV
in 199 5% FBS 5% Glycerol
1.30 x 10^4 Pfu's/ml in VERO cells
ATCC Designation No. VR-2516

6. CRIa
in 100 5% FBS 5% Glycerol
6.00 x 10^3 Pfu's/ml in VERO cells
ATCC Designation No. VR-2511

7. CaBCL
in 199 5% FBS 5% Glycerol
1.70 x 10^4 in VERO cells
ATCC Designation No. VR-2517

8. 19H 4MD
in 5% Glycerol
3.2 x 10^5 $TCID_{50}$/ml in MRC5 cells
ATCC Designation No. VR-2567.

9. 19H 3PI
in 5% Glycerol
4 x 10^5 $TCID_{50}$/ml in MRC5 cells
ATCC Designation No. VR-2564.

10. 19H 5MD
in 5% Glycerol
3.2 x 10^5 $TCID_{50}$/ml in MRC5 cells
ATCC Designation No. VR-2565.

11. CRIa MD
in 5% Glycerol
4 x 10^5 $TCID_{50}$/ml in MRC5 cells
ATCC Designation No. VR-2566.

12. 19HL 3PI
in 5% Glycerol
2.0 x 10^6 $TCID_{50}$/ml in MRC5 cells
ATCC Designation No. VR-2572.

13. wt 19
in 5% Glycerol
3.0 x 10^5 $TCID_{50}$/ml in MRC5 cells
ATCC Designation No. VR-2570.

14. WRSV
in 5% Glycerol
2.0 x 10^5 $TCID_{50}$/ml in MRC5 cells
ATCC Designation No. VR-2571.

B. The following depicts the passage status of exemplary derivative RSV strains of the present invention.

1. Line 19 MRC5 72-35°
2. Line 19 MRC5 24-25° st 1-33°
3. Line 19 MRC5 10-25° VERO 16-25° 6-20° 3-33°
4. Line 48 MRC 14-25° st 1-33° VERO 10-25° 1-33°
5. CRSV-BC5 CL17 MRC 30-25°
6. la-CRSV-5 CL15 MRC 27-25°
7. CRSV-BC13 MRC 19-25° 1-33°
8. Line 19 MRC5 P-70-35°, VERO P-3-35°, MRC5, P-2-35° clone 2 (3PI) plaque purified
9. Line 19 MRC5, P-92-35°, clone 5-1, purified by minimal limited dilution (MLD)
10. la-CRSV-5, MRC5, P-38-25°, MRC5, P-1-33°, purified by MLD
11. Line 19 HP clone 4-1, MRC5, P-103-33°, purified by MLD

12. Line 19 MRC 70-35°C, Vero P-35°C, large clone 6-35° (3PI), plaque purified

C. The following are the procedures for developing the attenuated deposited RSV strains of the present invention (see Specific Example 5 for additional details regarding the plaque purification). CRIa, CRIa MD, CaBCL and CaBCV were derived from WRSV. 19H, Ca19S, Ca19V, 19H 4MD, 19H 3PI, 19H 5MD and 19HL 3PI were derived from wt 19.

1. 19H

MRC5 cells were purchased in tubes from Bio Whittaker Laboratories. Media was removed. 1.2 ml EMEM + 5% FBS was added to each tube. 0.3 ml virus was added to each of 4 tubes. Tubes were incubated at 35°C and observed for development of cytopathic effect (CPE). Tubes were frozen at -70°C. Virus was harvested and passed to fresh cells.

2. Ca19S

The same steps as in 1. above were performed, except that tubes were passed 10 times at 35°C; 10 times at 30°C; 15 times at 25°C, and 2 times at 33°C.

3. Ca19V

The first 10 passages in MRC5 cells were performed as described in 1. above except that tubes were incubated at 25°C. Media was removed from 3 confluent 25cm^2 VERO flasks. Virus was diluted 1:5 in 1 x 199 + 5% FBS. 1 ml of virus was added to each of 2 flasks (1 flask is used as a control). Virus was adsorbed on a rocker at room temperature for 2 hours. 4 ml of 1 x 100 +5% FBS was added to each flask. Flasks were incubated at the appropriate temperature until 80% CPE was observed. Flasks were frozen at -70°C. Virus was harvested and passed to fresh cells. Virus was passed 16 times at 25°C; 6 times at 20°C; and, 2 times at 33°C.

4. a. Ca48V

The first 14 passages in MRC5 cells were performed as described in 2. above. Passage in VERO cells was performed as in 3. above. Virus was passed 10 times in VERO cells at 25°C and 1 time at 33°C.

5. CaBCV, CRIa and CaBCL

Same as 2. above except that 199 + 5% FBS was used.

An efficient plaque purification system was used (where indicated), which can evaluate individual plaques for temperature sensitivity. The following procedures were employed. Monolayers of Vero cells grown in 6- or 12-well plates were infected with 10-fold serial dilutions of virus in EMEM medium supplemented with 5% FBS and L glutamin. Each dilution was plated into three replicate wells. Virus was absorbed by incubation at 35°C on a rocker for 2 hours. The inoculum was removed and the cells were overlaid with 0.6% (W/V) Sea Kem ME agarose (final concentration) and 1xEMEM 3.5% FBS, L glutamine and gentamycin. The plates were allowed to solidify at room temperature and were incubated in CO_2 in parallel at 25°C, 33°C, 37°C and 39°C for 4 days. To clearly visualize developing plaques, a second overlay of agarose medium containing the same first overlay agarose medium with additional .01% (W/V) neutral red, was added on the fourth date after infection and plates were incubated in CO_2 at the appropriate temperature. Individual plaques were picked and were emulsified in 0.5 ml EMEM 5% FBS and either amplified or frozen at -70°C.

Each virus was evaluated by the time of plaque appearance, the plaque morphology, size and its characterization and titer. The growth of a given virus was expressed as (PFU/ml) for titration. The picked plaques were used to inoculate duplicate tubes containing Vero cell monolayers. One duplicate was incubated at 33°C and the other at 39°C up to 14 days. Cultures were checked for virus CPE. The tubes which demonstrated easily detectable CPE at 33°C, and no CPE at 39°C were selected for further plaque purification, titration and temperature sensitivity studies.

Conventional minimal limited dilution procedures, known to those skilled in the art, were followed where indicated.

SPECIFIC EXAMPLE 2 - TEMPERATURE SENSITIVITY STUDIES

To screen the cold-adapted and high passage virus for the presence of temperature sensitive (*ts*) variants, viruses were tested at 39°C, 37°C and 33°C by one of two methods of titration: plaque immunoassay or $TCID_{50}$.

Results

Line 19H is *ts* when assayed in both MRC cells and under agarose in Vero cells. Line Ca19S has a 5 log reduction in growth at 39°C versus that at 33°C. Line CRla is also *ts* in MRC cells. WRSV grows as well at 39°C as it does at 33°C.

Lines Ca19V and Ca48V are both *ts* when assayed in Vero cells using the second antibody technique and under agarose. Line Ca19V has a 5 log reduction in

growth at 39°C versus that at 33°C and line Ca48V has a 3 log reduction in growth at the non-permissive temperature.

The following tables further set forth the results of the temperature sensitivity study.

Table 2

$TCID_{50}$ in MRC Cells on Day 14

Virus Lines	33°C	37°C	38°C	39°C
19H	1.00×10^8			3.16×10^5
19H	4.68×10^6	2.15×10^5		4.68×10^4
Ca19S	3.16×10^7		3.16×10^3	3.16×10^2
CRla	4.68×10^3		4.68×10^2	3.16×10^1
WRSV	3.16×10^4		3.16×10^4	1.00×10^4

Table 3

Pfu's in Vero Cells on Day 7

Virus Lines	33°C	37°C	38°C	39°C
Ca19V	3.16×10^{10}		5.85×10^9	2.54×10^5 pinpoint plaques
Ca48V	2.50×10^7	8.00×10^6		6.00×10^4 pinpoint plaques

Materials and Methods

$TCID_{50}$ in MRC5 Cells. Virus to be titrated was diluted 10^{-1} in EMEM + 5% FBS. Confluent MRC5 tubes were used, for each dilution and for each temperature (total of 96 tubes for 3 temperatures). 1 ml of a viral dilution was added to each tube. Tubes were incubated at 33°C, 37°C or 38°C, and 39°C. Tubes were read daily to day 14 for CPE. $TCID_{50}$ was calculated using the method of Reed and Muench.

Plaque Immunoassay - Pfu's in HEP2 Cells or Vero Cells. HEP2 Cells (or VERO cells) were grown in a 12 well microtiter plate until semi-confluent and media was removed. Virus was diluted in 1 x 199 + 5% FBS 10^{-1} to 10^{-7}. Cells were inoculated in triplicate, 0.5 ml/well and allowed to adsorb at 35°C for 2 hours. Inoculum was then removed. The cells were overlaid with 2 ml of the 1:1 mixture of

2 x EMEM with 6% FBS and the 4% Methyl Cellulose (4 gm Methyl Cellulose and 100 ml Type I Deionized Water, autoclaved to sterilize; final concentration 2% Methyl Cellulose) and incubated at 35°C with 5% CO_2 for 7 days. Overlay media was then discarded. The cells were fixed with cold 80% methanol at -70°C for 1 hour. The methanol was then removed and the plates were frozen at -70°C. Plates were allowed to thaw at room temperature. 1 ml of 5% Blotto media (25 gm Milk and 500 ml PBS) was added to each well and the wells were incubated at 35°C for 30 minutes. 5% Blotto media was then removed and 1 ml of 5% Blotto media with 1/100 dilution of anti-RSV antibody was added. Incubation at 35°C for 30 minutes took place. After incubation, 5% Blotto media with antibody was removed and cells were washed with 5% Blotto media. 1 ml of 5% Blotto media with 1/100 dilution of conjugate antibody was then added and incubation took place at 35°C for 30 minutes. 5% Blotto media with conjugate antibody was removed and cells were washed with PBS. 1 ml of 1:1 mix of peroxide solution substrate (4 chloro-1 napthol) + H_2O_2 was added and incubation took place at room temperature for 1-5 minutes. During this period color development was watched carefully. Cells were then washed with PBS. Plaques were counted and Pfu's recorded.

SPECIFIC EXAMPLE 3 - IMMUNOGENICITY STUDIES

A. Immunogenicity of RSV Lines (Study 1)

A study was performed to determine the immunogenicity of line 19H. Pathogen-free BALB/c mice (approximately 8 weeks old) were immunized intranasally with either 1.6 X10^6 $TCID_{50}$ of 19H, 2.5 X 10^5 pfu of A2 mouse adapted virus (designated live virus), or 5% glycerol (designated placebo). Animals were bled 4 weeks after the primary inoculation and boosted at 4 weeks with an equivalent dose of the vaccine formulation. Serum samples were also taken 4 weeks after the booster dose. Anti-F antibody titer was determined as follows: immunoaffinity purified RSV-F antigen was coated on wells of Nunc-immuno Maxi Sorp flat bottom microtiter plates, by incubating antigen overnight at room temperature in 0.05M Carbonate - Bicarbonate buffer, pH 9.6. Wells were blocked for non-specific binding by adding 0.1% BSA in PBS for 30 min. at room temperature, followed by two washes in a washing buffer of 0.1% BSA in PBS + 0.1% Tween 20. Mice sera was diluted in two or four-fold serial dilutions, and added to wells. After 1 hour incubation at room temperature, plates were washed five times with washing buffer, and horseradish peroxidase labeled conjugate was added at the appropriate optimal dilution in washing buffer. The total IgG assay used $F(ab')_2$, goat anti-mouse IgG (H+L specific)-HRP

from Jackson Immuno Research Laboratory Inc., Baltimore. The IgG 1 assay used sheep anti-mouse IgG1-HRP from Serotec. The IgG 2a and IgA assays used goat anti-mouse IgG 2a-HRP and goat-anti-mouse IgA-HRP respectively, from Caltag Laboratories, San Francisco. Following 1 hour incubation at room temperature, the plates were washed five times with washing buffer, and a substrate hydrogen peroxide in the presence of tetramethylbenzidine was added. The color reaction was stopped by adding 2M sulfuric acid. The color was read in a Multiscan Titertek plate reader at an optical density (OD) of 450nm. The titre was taken as the reciprocal of the last dilution at which the OD was approximately double. This OD must be greater than the negative control of the assay at the starting dilution. The pre-immune serum of each animal was used as the negative control.

Line 19H elicited levels of anti-F IgG antibodies that were equivalent to those induced by the A2 mouse adapted virus (see Figures 1A, 1B and 1C). Sera from animals that received two doses of line 19H had a balanced anti-RSV F Ig_1/IgG_{2a} response. As outlined in the table below, the sera of animals that were immunized with two doses of 19H had RSV-specific neutralizing antibodies that were comparable to those obtained following inoculation with live mouse adapted RSV. Thus, line 19H was immunogenic in the mouse model.

Table 4

Serum antibody response of BALB/c mice immunized with *ts* mutant

Formulation	**Neutralization titer[a,b] ($\log_2 \pm$ s.d.)**	
	4 Week Bleed	**8 Week Bleed**
19H	8.2 ± 0.9	9.5 ± 1.2
Live RSV (mouse-adapted A2 virus)	8.5 ± 0.8	10.6 ± 0.7
Placebo	<3.3 ± 0.0	<3.3 ± 0.0

[a]Neutralization titer determined by complement-enhanced 60% plaque-reduction assay
[b]Each value represents the reciprocal mean titer of at least 6 animals

B. Immunogenicity of RSV Lines (Study 2)

A second study was performed to determine the immunogenicity of the following RSV lines: WRSV (Vero $35°_1$, titer 2.00×10^6 Pfu's/ml in Vero cells), CRla, CaBCV, 19H, Ca19V and Ca48V. Balb/c/AnNTacfBR three week old male mice from Taconic, 272 Hoover Avenue, Germantown, NY 12526, were used. Mice were

anesthetized IP with 200 μl ketoset diluted 1:10 and inoculated IN with 50 μl undiluted virus, 6 mice/group. Mice were boosted with 50 μl virus diluted 1:2 in 199 media. Approximately one week later, mice were bled for serum. Neutralization titers were done in VERO cells.

All of the lines tested for neutralization antibodies had titers of at least 1:20. Line 19H had a titer of 1:80, however, WRSV had a titer of 1:320. The following tables further set forth the results of the immunogenicity study.

Table 5

Neutralization Titers (60% Reduction) without Complement†

Antiserum to Virus	Titers
19H	1:80
CRIa	1:40
Ca19V	1:20
CaBCV	1:40
Ca48V	1:40
WRSV	>1:320
Normal Serum	<1:20

†Serum periodate and heat treated (56° for 1 hour); serum diluted in presence of 2.5% FBS.

Table 6

ELISA Titers*

Antiserum to Virus	Titers⁺
19H	1:1280
CRIa	1:320
Ca19V	1:1280
CaBCV	1:2560
Ca48V	neg
WRSV	1:640

*Antigen - Whole RSV Virus
⁺Titers corrected for normal serum

C. **Immunogenicity Of RSV Lines (Study 3)**

A third study was performed to determine the immunogenicity of the following RSV lines: Ca19S, 19H, Ca19V, Ca48V, WRSV and Line 19 MRC5 28 25° (not deposited). Mice were infected with wild type and the RSV lines with twice boosted procedures, similar to the procedure described above. As shown in the table below, serum neutralization titers, carried out in the presence of complement, were reported as the reciprocal of the last dilution which reduced the number of viral plaques by 60%. Also shown in the table below, ELISA titers were reported as the reciprocal of the last dilution which had an OD reading of 0.1 or greater after correction for normal serum. All the lines tested for neutralization antibodies had titers of at least 320 and ELISA titers ranging from 160 to 40,960 after the second boost. Although line Ca19S produced the highest neutralization titer, all lines were similar in neutralizing ability and reached peak titer after the first boost. Line Ca19V produced the highest ELISA IgG titer. All RSV attenuated lines were immunogenic.

Table 7

Neutralization and ELISA Titers of Mice Immunized with Vaccine Lines

Viruses[a]	Titers Of Inoculum	Neutralization[b]			ELISA IgG[c]		
		2 Weeks[d]	4 Weeks	6 Weeks	2 Weeks	4 Weeks	6 Weeks
WRSV	5.3	80	640	320	160	2560	80
Line 19 MRC5 28 25°	5.7	80	640	1280	160	640	1280
Ca19S	7.5	80	640	640	320	2560	1280
19H	7.3	80	320	320	80	2560	320
Ca19V	6.3	320	640	640	320	5120	40960
Ca48V	7.4	80	320	320	160	5120	10240

[a]Viruses used were not plaque purified.
[b]Reported as reciprocal of last dilution which reduced viral plaques by 60%.
[c]Reported as reciprocal of last dilution which had an OD of 0.1 or greater after adjustment for normal serum.
[d]Weeks after Initial Vaccination.

D. Immunogenicity Of RSV Lines (Study 4)

A fourth study was performed to determine the immunogenicity of the following plaque purified RSV lines: 19HL 3PI, 19H 3PI, 19H MD and CRla MD. Pathogen-free BALB/c mice (approximately 8 weeks old) were immunized intranasally with either 2 X 10^4 $TCID_{50}$ of the lines or their respective progenitor viruses (designated wt 19 and WRSV), 2.0 X 10^4 pfu of mouse-adapted virus (designated live virus), or medium +5% FBS +5% glycerol (designated placebo). Animals were bled 4 weeks after the primary inoculation and boosted at 4 weeks with an equivalent dose of the vaccine formulation. Serum samples were also taken 4 weeks after the booster dose. All plaque purified lines elicited anti-F IgG antibodies at 4 and 8 weeks (see Figures 2A and 2B, wherein each value represents the mean titer of antisera from 6 animals). At the 8 week time point, the sera of animals that had received 2 doses of the various plaque purified lines had anti-RSV F IgG antibody titres that were comparable to that observed in the sera of mice that were immunized with the mouse-adapted virus (designated live virus). As shown in Figure 3 (values represent the mean titer of antisera from 6 animals), the sera of animals that were immunized with two doses of the various plaque purified lines had high levels of RSV-specific neutralizing antibodies. Thus, all RSV attenuated lines tested were immunogenic in the mouse model.

E. Boosting By Alternative Route of Administration

A study was performed to determine the effect of boosting by a route of administration that differs from the initial inoculation route of administration. Taconic Balb/c/AnNTacfBR 3 week old male mice were anesthetized IP with 200 μl ketoset diluted 1:10 in PBS and inoculated IN with 50 μl undiluted virus, WRSV (VERO 35°, Titer 2.00 x 10^6 Pfu's/ml in VERO cells), CRla, CaBCV, 19H and Ca48V. Mice were boosted with 100 μl virus via footpad injection. Approximately three weeks later, mice were boosted with 200 μl half virus and half complete Freund's adjuvant intramuscularly. Approximately ten days later, mice were bled for serum. Neutralization titers were done in VERO cells in the presence of complement. The following table sets forth the results of the study.

Table 8

Neutralization Data in Presence of Complement (60% Reduction)

Antiserum to:	Titer with Complement*
Normal Serum	
19H	1:160
CaBCV	1:160
Ca48V	>1:640
CRIa	>1:640
WRSV	>1:640

*Titer reported as last dilution with 60% reduction over positive controls with complement average positive control = 100.33 colonies/well.

F. Cytotoxicity Study

Generation of CTL. Spleens from two BALB/c mice from each group that were immunized with either live mouse adapted A2 virus, line 19H or placebo, (see A. above, Immunogenicity of RSV Lines (Study 1)), were removed three weeks after the booster dose. Single cell suspensions were prepared and incubated at 2.5×10^7 cells in RPMI 1640 plus 10% FBS. Gamma-irradiated (3,000 rads) syngeneic spleen cells were infected with RSV at an MOI of 1 for 2 h. The cells were washed twice to remove free virus and 2.5×10^7 spleen cells in a final volume of 10 ml of complete medium. CTL activity was tested 5-6 days following re-stimulation.

Cytoxicity assay. On the date of the assay, effector cells were washed twice with fresh medium and viable cell counts were determined by the Trypan blue dye exclusion method. BC cells (2×10^6 cells), a BALB/c fibroblast cell line, as well as BCH4 cells (2×10^6 cells), a BALB/c fibroblast T cell line persistently infected with RSV, were pulsed with 200 μCi of Sodium ^{51}chromate (Dupont) for 90 min. The targets were washed three times with medium to remove free ^{51}chromium. Viable cell counts of the target cells were determined and target cell suspensions were prepared at 2×10^4 cells/mL. Washed responder T-cells (in 100 μl) were incubated with 2×10^3 target cells (in 100 μl) at various Effector:Target cell ratios in triplicate in 96-well V-bottomed tissue-culture plates for 4 h at 37°C with 6% CO_2. Spontaneous and total release of ^{51}chromium were determined by incubating target cells with either medium or 2.5% Triton-X100 in the absence of responder lymphocytes. Six replicates of each were prepared. After 4 h plates were centrifuged at 200 x g for 2 min and 100 μl supernatant was removed from each well to determine the amount of ^{51}chromium released. Percentage specific ^{51}chromium release was calculated as (Experimental Release - Spontaneous Release) (Total Release - Spontaneous Release) x 100. The

Spontaneous Release of ^{51}chromium in the absence of effector cells was found to be between 10-15% in these studies.

Figure 4 shows the results of the study. In Figure 4, lysis of BC (filled symbols) and BCH4 (empty symbols) by CTL generated from BALB/c mice immunized with placebo (Triangle), live RSV (Square) or line 19H (Circle) is shown. Mice immunized with live RSV (empty square) or 19H (empty circle) lysed BCH4 cells (RSV infected) significantly at all effector to target cell ratios when compared to the lysis of BC (un-infected) cells. There was no significant levels of lysis by effector cells from the placebo indicating that line 19H is capable of inducing significant levels of CTL activity.

G. Protection Study

Generation of CTL. Spleens from two BALB/c mice from each group that were immunized with either the mutants or their respective progenitor viruses, live mouse adapted virus or medium (placebo) were removed three weeks after the booster dose. Single cell suspensions were prepared and incubated at 2.5 X 10^7 cells in RPMI 1640 plus 10% FBS. Gamma-irradiated (3,000 rads) syngeneic spleen cells were infected with RSV at an MOI of 1 for 2h. The cells were washed twice to remove free virus and 2.5 X 10^7 spleen cells in a final volume of 10 mL of complete medium. CTL activity was tested 5-6 days following re-stimulation.

Cytotoxicity assay. On the day of the assay, effector cells were washed twice with fresh medium and were resuspended in 2 mL of complete medium. BC cells (2 X 10^6 cells), a BALB/c fibroblast cell line, as well as BCH4 cells (2 X 10^6 cells), a BALB/c fibroblast T cell line persistently infected with RSV, were pulsed with 200μCi of Sodium ^{51}chromate (Dupont) for 90 min. The targets were washed three times with medium to remove free ^{51}chromium. Viable cell counts of the target cells were determined and target cell suspensions were pared at 2 X 10^4 cells/mL. Washed responder T-cells at various dilutions (in 100 μl) were incubated with 2 X 10^3 target cells (in 100 μl) in triplicate in 96-well V bottomed tissue-culture plates for 4 h at 37°C with 6% CO_2. Spontaneous and total release of ^{51}chromium were determined by incubating target cells with either medium of 2.5% Triton -X100 in the absence of responder lymphocytes. Six replicates of each were prepared. After 4 h plates were centrifuged at 200 X g for 2 min. and 100μl of supernatant was removed from each well to determine the amount of ^{51}chromium related. Percentage specific ^{51}chromium release was calculated as (Experimental Release-Spontaneous Release)/ (Total release - Spontaneous release) X 100. The spontaneous release of ^{51}chromium in

the absence of effector cells was found to be between 10-15% in these studies. The lysis in cultures is directly proportional to the number of effector cells present in the culture, which in turn is proportional to the number of CTL precursors activated *in vivo* by that particular immunogen.

The results of this cytotoxicity study are shown in Figure 5. In Figure 5, lysis of BC (open symbols) and BCH4 cells (filled symbols) by CTL generated from BALB/c mice immunized with either placebo, 19HL 3PI, 19H 3PI, 19H MD, wt 19, CRIa MD or WRSV, or live mouse adapted virus is shown. Mice immunized with live mouse adapted RSV, 19HL 3PI, 19H 3PI, 19H MD, CRIa MD, wt 19 and WRSV, lysed BCH4 cells (RSV infected) at all effector cell dilutions when compared to the lysis of BC (non-infected) cells. There were no significant level of lysis by effector cells from the placebo indicating that all the tested viruses are capable of inducing significant levels of CTL activity.

To evaluate the ability of the plaque purified deposited viruses to protect mice against live virus challenge, mice that were immunized with either the plaque purified viruses, progenitor viruses or medium alone (see D. above, Immunogenicity of RSV Lines (Study 4)), were challenged with 10^6 pfu of RSV A2 immediately after the 8 week bleed. Lungs were harvested four days after virus challenge and virus titers in lung homogenates were determined by the plaque assay. As shown in the table below, mice immunized with the viruses of the present invention were protected against live virus challenge. The protective ability was comparable to that observed with mice that were inoculated with live mouse adapted virus.

Table 9

Protective Ability Of The Plaque Purified Viruses And Progenitor Viruses

Virus	Mean Virus Lung Titre (log pfu/g ± s.d.)*	% Animals Protected
Placebo	5.2 ± .06	0
19HL 3PI	≤ 1.7 ± 0	100
19H 3PI	≤ 1.7 ± 0	100
19H MD	≤ 1.7 ± 0	100
wt 19	≤ 1.7 ± 0	100
CRIa MD	≤ 1.7 ± 0	100
WRSV	1.9 ± 2.2	50
Live virus	≤ 1.7 ± 0	100

*Represents the mean value of 6 animals.

SPECIFIC EXAMPLE 4 - SEQUENCE ANALYSIS OF THE F GENE

A. Sequence Comparison - WRSV

In identifying the molecular basis for the *ts* phenotype, the F gene of the wild type (WRSV), line Ca19V and line 19H were sequenced using polymerase chain reaction (PCR). The F gene is composed of 1899 nucleotides, 13 of which are non-coding at the 3′ end. Both viruses were grown in Vero cells to isolate the RNA for sequencing. Comparison of the F genes of line Ca19V and line 19H revealed 73 nucleotide and 15 amino acid differences. Comparison of the F genes of line 19H and WRSV revealed 72 nucleotide and 13 amino acid differences. There are 11 nucleotide changes and 6 amino acid changes between the F genes of the two line 19 attenuated viruses, Ca19V and 19H. Only base changes (no insertions or deletions) were found. The F genes of the two attenuated line 19 viruses have 66 nucleotides and 11 amino acids in common but differ from that of WRSV (amino acid positions 66, 76, 79, 97, 119, 129, 191, 357, 384, 522 and 530).

The Garnier Osguthorpe Robson (GOR) predicted F protein structures of the two line 19 viruses are nearly identical; however, the GOR F protein structure of WRSV differs at amino acid 97, 119, 191, 357 and 522 from both attenuated viruses and differs at amino acid 294 only from line Ca19V. Amino acid 97 (threonine in both line 19's and methionine in WRSV) predicts a turn in the attenuated line 19 viruses

not present in WRSV. Amino acid 119 (phenylalanine in both line 19's and leucine in WRSV) predicts an additional turn in the attenuated line 19 viruses not present in WRSV. Amino acid 191 (lysine in the attenuated lines 19 viruses and arginine in WRSV) predicts the formation of an alpha helix in the attenuated line 19 viruses while WRSV continues a beta sheet fold and then turns. Amino acid 357 predicts the formation of an alpha helix in WRSV not present in either attenuated line 19 virus and amino acid 522 predicts a turn in WRSV not present in either attenuated line 19 viruses. Amino acid 294 predicts the formation of a beta sheet in WRSV and 19H not present in Ca19V. It is interesting that, although there are 6 amino acid differences between the two attenuated line 19 viruses, the 2 attenuated viruses have the same predicted protein fold whereas the WRSV fold is quite different. Thus, amino acids 97, 119, 191, 357 and 522 are good candidates for attenuating lesions in the F protein.

Table 10

Sequence Comparison of the Genes Coding for the F Proteins of WRSV, Ca19V and 19H

Nucleo-tide #	WRSV	Ca19V	19H	Amino Acid #	WRSV	Ca19V	19H
76	C	T	T				
85	T	C	C				
106	A	G	G				
131	***G***	***G***	***A***	***40***	***Val***	***Val***	***Ile***
155	C	T	T				
199	T	C	C				
209	A	G	G	66	Lys	Glu	Glu
220	T	C	C				
235	T	C	C				
240	T	C	C	76	Val	Ala	Ala
250	G	A	A	79	Met	Ile	Ile
265	T	C	C				
271	T	C	C				
296	T	C	C				
303	T	C	C	<u>97</u>	<u>Met</u>	<u>Thr</u>	<u>Thr</u>
322	A	T	T				
352	***G***	***G***	***A***				

Nucleo-tide #	WRSV	Ca19V	19H	Amino Acid #	WRSV	Ca19V	19H
368	C	T	T	119	Leu	Phe	Phe
370	C	T	T				
398	T	A	A	129	Leu	Ile	Ile
430	T	C	C				
457	C	T	T				
505	G	A	A				
511	G	A	A				
514	C	A	A				
541	G	A	A				
585	G	A	A	191	Arg	Lys	Lys
604	C	T	T				
622	G	A	A				
623	T	C	C				
716	***A***	***G***	***C***	***235***	***Arg***	***Gly***	***Arg***
718	G	A	A				
763	C	T	T				
787	G	A	A				
871	C	T	T				
893	***G***	***A***	***G***	***294***	***Glu***	***Lys***	***Glu***
898	A	G	G				
906	***C***	***C***	***A***	***298***	**Ala**	***Ala***	***Glu***
959	T	C	C				
961	A	G	G				
1003	A	C	C				
1015	A	G	G				
1048	T	C	C				
1057	A	T	T				
1083	A	C	C	357	Lys	Thr	Thr
1087	T	C	C				
1090	A	G	G				
1116	***A***	***T***	***A***	***368***	***Asp***	***Val***	***Asp***

Nucleo-tide #	WRSV	Ca19V	19H	Amino Acid #	WRSV	Ca19V	19H
1126	C	T	T				
1163	G	A	A	384	Val	Ile	Ile
1180	***C***	***C***	***T***				
1206	***C***	***T***	***C***	***398***	***Ser***	***Leu***	***Ser***
1222	***C***	***T***	***C***				
1228	C	T	T				
1241	*C*	*C*	*T*				
1246	A	G	G				
1465	C	A	A				
1501	T	C	C				
1504	G	A	A				
1519	T	C	C				
1520	T	C	C				
1576	A	C	C				
1577	A	G	G	<u>522</u>	<u>Thr</u>	<u>Ala</u>	<u>Ala</u>
1603	A	G	G	530	Ile	Met	Met
1655	***C***	***T***	***C***				
1669	C	A	A				
1705	G	A	A				
1739	A	T	T				
1751	C	T	T				
1781	C	A	A				
1802	G	A	A				
1813	T	C	C				
1829	T	C	C				
1838	A	G	G				
1841	C	T	T				
1843	T	C	C				
1847	T	C	C				

Differences between Ca19V and 19H are italicized and bolded. Amino acids which impact on the predicted protein folds are underlined.

B. **Sequence Comparison - wt 19**

The nucleotide sequence of the F genes of line 19 wild type (wt 19), Ca19V and 19H, were compared by *f-mol* sequencing. The following table lists the nucleotide and amino acid changes in the F genes between lines Ca19V, 19H and wt 19.

Table 11

Sequence Differences Between The F Genes Of wt 19, Ca19V and 19H

Nucleo-tide #	wt 19	19H	Ca19V	Amino Acid #	wt 19	19H	Ca19V
131	G	**A**	G	40	val	**iso**	val
352	A	A	*G*				
716	C	C	*G*	235	arg	arg	*gly*
893	G	G	*A*	294	glu	glu	*lys*
906	C	**A**	C	298	ala	**glu**	ala
1116	A	A	*T*	368	asp	asp	*val*
1180	T	T	*C*				
1206	C	C	*T*	398	ser	ser	*leu*
1222	T	**C**	T				
1241	T	T	*C*				
1249	C	**A**	*A*				
1655	C	C	*T*				

Differences between 19H and wt 19 are bolded.
Differences between Ca19V and wt 19 are italicized.

Between wt 19 and 19H, there were 4 nucleotide differences, 2 of which coded for amino acid differences. Amino acid 40, valine in wt 19 and isoleucine in 19H, is a conserved change since both are hydrophobic and neither is charged. The difference at amino acid 298 is not conserved. Alanine, in wt 19, is hydrophobic and not charged while glutamic acid in 19H, is not hydrophobic and is negatively charged. Chou Fasman analysis predicts that the glutamic acid of 19H extends an alpha helix thus postponing the formation of a beta sheet predicted by the alanine at 298 of the wt 19 F protein.

Between wt 19 and Ca19V, there are 9 nucleotide differences and 4 amino acid differences, none of which is a conserved change. Amino acid 235 is basic arginine to uncharged glycine; amino acid 294 is negatively charged glutamic acid to positively charged lysine; amino acid 368 is negatively charged aspartic acid to hydrophobic valine; and amino acid 398 is uncharged serine to hydrophobic leucine. The structure of the two F proteins, as predicted by Chou Fasman, differs only at amino acid 234; Ca19V extends an alpha helix, thus losing a turn predicted for the wt 19 F protein.

None of the amino acid differences is shared by the two line 19 attenuated viruses, but nucleotide 1249 (which does not code for an amino acid change) is C in wt 19 and A in both Ca19V and 19H.

SPECIFIC EXAMPLE 5 - PLAQUE PURIFICATION

As indicated above, several of the deposited strains were plaque purified. In particular, Line 19HL 3PI was passed 72 times in MRC5 cells, then plaque purified three times in Vero cells. Line 19H 3PI was passed 70 times in MRC5 cells, then plaque purified three times in Vero cells and passed 2 times in MRC5 cells. Line 19H MD was passed 92 times in MRC5 cells, then purified by limiting dilution three times. Line CRIa MD was passed 28 times at 25°C and 1 time at 33°C then purified by limiting dilution five times. The following table sets forth the titers of the strains at 33°C and 39°C, illustrating temperature sensitivity.

Table 12

$TCID_{50}$ In MRC5 Cells

Virus	33°C	39°C
19HL 3PI	1.0×10^6	1.0×10^5
19H 3PI	3.2×10^5	2.0×10^3
19H MD	3.2×10^5	1.0×10^3
CRIa MD	$>1.0 \times 10^6$	2.0×10^3
19H	3.16×10^6	
19H 4MD	3.16×10^4	
WRSV	2.0×10^5	2.0×10^5

SPECIFIC EXAMPLE 6 - HUMAN STUDIES

The attenuated virus of the present invention is administered to human subjects according to well established human RSV protocols, for example, those described in Wright et al., *Infect. Immun.* 37:397-400 (1982); Kim et al., *Pediatrics* 52:56-63 (1973) and Wright et al., *J. Pediatr.* 88:931-936 (1976). Briefly, adults or children are inoculated intranasally via droplet with 10^2 to 10^9 PFU, preferably 10^4 to 10^5 PFU, of attenuated virus per ml in a volume of 0.5 ml. Antibody response is evaluated by complement fixation, plaque neutralization, and/or enzyme-linked immunosorbent assay. Individuals are monitored for signs and symptoms of upper respiratory illness. Subsequent immunizations are administered periodically to the individuals as necessary to maintain sufficient levels of protective immunity.

The foregoing discussion discloses and describes merely exemplary embodiments of the present invention. One skilled in the art will readily recognize from such discussion, and from the accompanying claims, that various changes, modifications and variations can be made therein without departing from the spirit and scope of the invention as defined in the following claims.

All patents and other references cited herein are expressly incorporated by reference.

WE CLAIM:

1. A respiratory syncytial virus selected from the group consisting of the viruses having ATCC Designation Nos. VR-2511, VR-2512, VR-2513, VR-2514, VR-2515, VR-2516, VR-2517, VR-2564, VR-2565, VR-2566, VR-2567 and VR-2572, and derivative viruses thereof.

2. The attenuated virus of Claim 1 for use as an active pharmaceutical substance.

3. The use of the attenuated virus of Claim 1 for the preparation of a medicament for the treatment or prevention of disease caused by infection by respiratory syncytial virus.

4. A method of producing a vaccine against disease caused by infection by respiratory syncytial virus, comprising:

a) administering the virus of Claim 1 to a test host to determine an amount and a frequency of administration thereof to elicit a protective immune response in said host; and

b) formulating said virus in a form suitable for administration to a treatable host in accordance with said determined amount and frequency of administration.

5. A method of determining the presence of antibodies specifically reactive with a respiratory syncytial virus in a sample comprising the steps of:

a) contacting the sample with the virus of Claim 1 to produce complexes comprising the virus and antibodies present in the sample specifically reactive therewith; and

b) determining production of the complexes.

6. A method of determining the presence of a respiratory syncytial virus in a sample comprising the steps of:

a) contacting the sample with an antibody specifically reactive with a respiratory syncytial virus of Claim 1 to produce complexes comprising the antibody and the virus present in the sample specifically reactive therewith; and

b) determining production of the complexes.

7. A nucleic acid molecule encoding the respiratory syncytial virus of Claim 1 and equivalent nucleic acid molecules thereof.

8. A vaccine composition comprising an attenuated respiratory syncytial virus and a pharmaceutically acceptable carrier.

9. The vaccine composition of Claim 8, wherein the attenuated respiratory syncytial virus is selected from the group consisting of the viruses of Claim 1 and derivative viruses thereof.

10. A method of immunizing a host against disease caused by infection by respiratory syncytial virus which comprises administering to the host an immunoeffective amount of the vaccine of Claim 8 or the nucleic acid molecule of Claim 7.

11. An attenuated respiratory syncytial virus characterized by having a codon encoding an amino acid in the F protein chosen from the group consisting of a codon encoding isoleucine at amino acid 40, a codon encoding glutamic acid at amino acid 66, a codon encoding alanine at amino acid 76, a codon encoding isoleucine at amino acid 79, a codon encoding threonine at amino acid 97, a codon encoding phenylalanine at amino acid 119, a codon encoding isoleucine at amino acid 129, a codon encoding lysine at amino acid 191, a codon encoding glycine at amino acid 235, a codon encoding arginine at amino acid 235, a codon encoding lysine at amino acid 294, a codon encoding glutamic acid at amino acid 294, a codon encoding glutamic acid at amino acid 298, a codon encoding alanine at amino acid 298, a codon encoding threonine at amino acid 357, a codon encoding valine at amino acid 368, a codon encoding aspartic acid at amino acid 368, a codon encoding isoleucine at amino acid 384, a codon encoding leucine at amino acid 398, a codon encoding serine at amino acid 398, a codon encoding alanine at amino acid 522 and a codon encoding methionine at amino acid 530.

12. An attenuated respiratory syncytial virus characterized by having a codon encoding an amino acid in the F protein chosen from the group consisting of a codon encoding isoleucine at amino acid 40, a codon encoding glycine at amino acid 235, a codon encoding arginine at amino acid 235, a codon encoding lysine at amino acid 294, a codon encoding glutamic acid at amino acid 294, a codon encoding glutamic acid at amino acid 298, a codon encoding alanine at amino acid 298, a codon encoding valine at amino acid 368, a codon encoding aspartic acid at amino acid 368, a codon encoding leucine at amino acid 398 and a codon encoding serine at amino acid 398.

13. A respiratory syncytial virus selected from the group consisting of wt 19, and WRSV, and derivative viruses thereof.

14. The virus of Claim 13, wherein the derivative virus is attenuated.

15. The attenuated virus of Claim 14 for use as an active pharmaceutical substance.

16. The use of the attenuated virus of Claim 14 for the preparation of a medicament for the treatment or prevention of disease caused by infection by respiratory syncytial virus.

17. A method of producing a vaccine against disease caused by infection by respiratory syncytial virus, comprising:

a) administering the virus of Claim 14 to a test host to determine an amount and a frequency of administration thereof to elicit a protective immune response in said host; and

b) formulating said virus in a form suitable for administration to a treatable host in accordance with said determined amount and frequency of administration.

18. A method of determining the presence of antibodies specifically reactive with a respiratory syncytial virus in a sample comprising the steps of:

a) contacting the sample with the virus of Claim 14 to produce complexes comprising the virus and antibodies present in the sample specifically reactive therewith; and

b) determining production of the complexes.

19. A method of determining the presence of a respiratory syncytial virus in a sample comprising the steps of:

a) contacting the sample with an antibody specifically reactive with a respiratory syncytial virus of Claim 14 to produce complexes comprising the antibody and the virus present in the sample specifically reactive therewith; and

b) determining production of the complexes.

20. A nucleic acid molecule encoding the respiratory syncytial virus of Claim 13 and equivalent nucleic acid molecules thereof.

21. The vaccine composition of Claim 8, wherein the attenuated respiratory syncytial virus is selected from the group consisting of the viruses of Claim 14 and derivative viruses thereof.

22. A method of immunizing a host against disease caused by infection by respiratory syncytial virus which comprises administering to the host an immunoeffective amount of the vaccine of Claim 21 or the nucleic acid molecule of Claim 20.

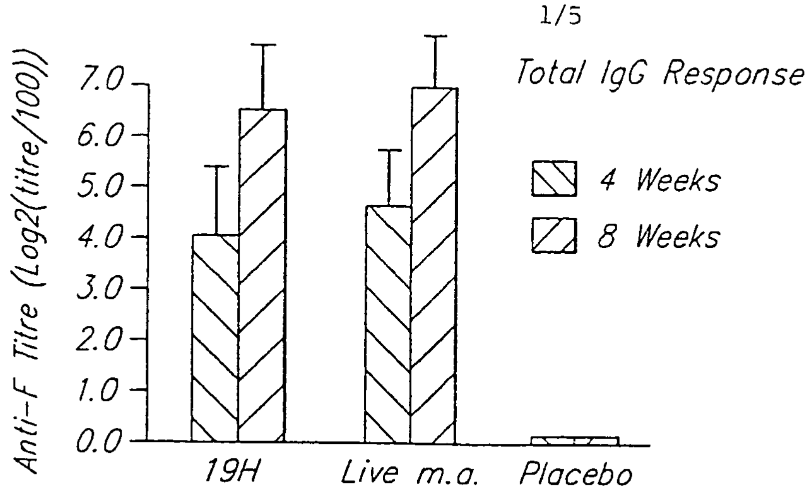


FIG. 1A.

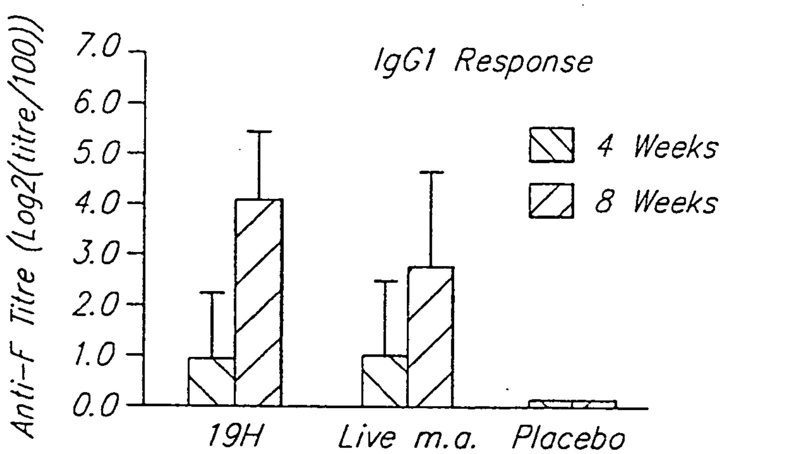
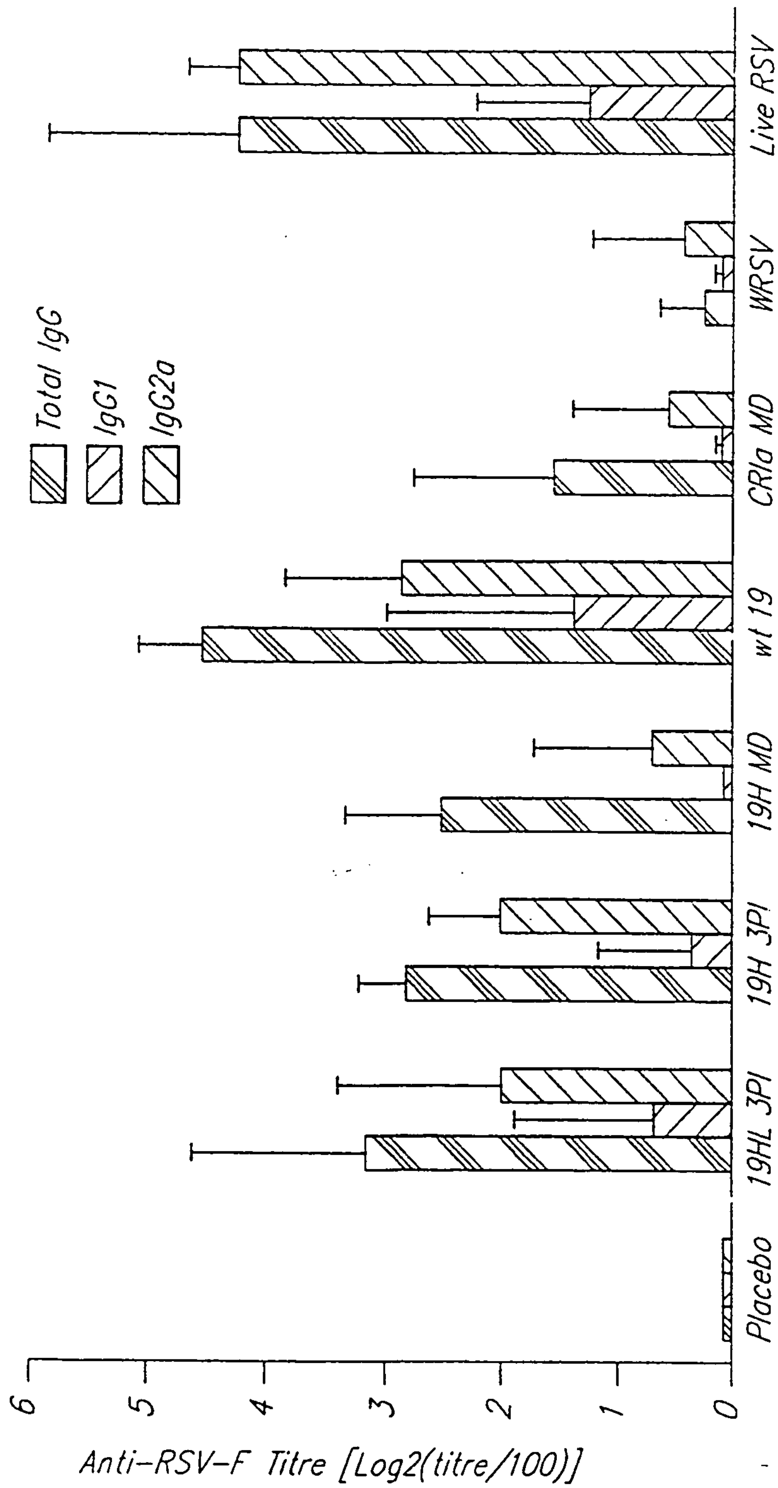


FIG. 1B.

FIG. 1C.



Total IgG
IgG1
IgG2a
Live RSV
WRSV
CRIa MD
wt 19
19H MD
19H 3PI
19HL 3PI
Placebo
0
1
2
3
4
5
6
Anti-RSV-F Titre [Log2(titre/100)]

FIG. 2A.

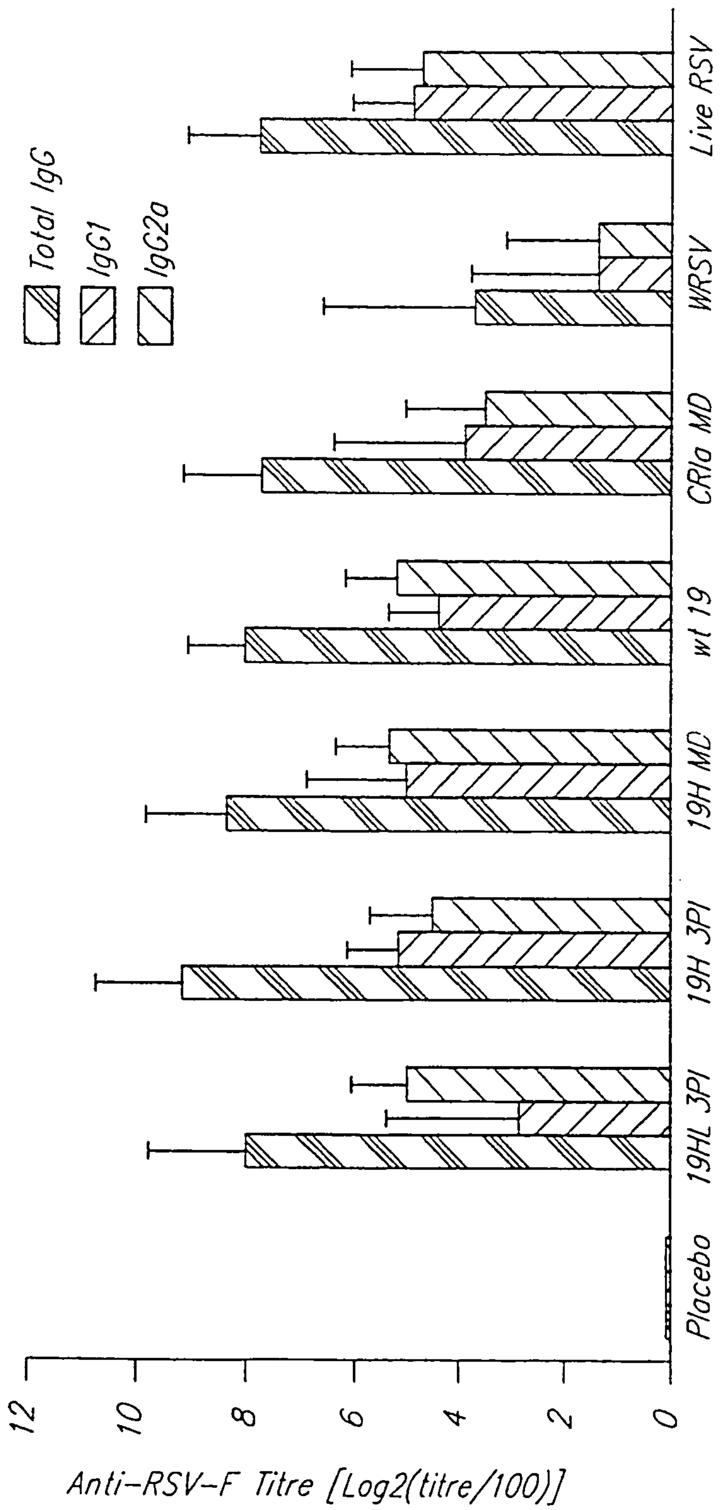

Total IgG
IgG1
IgG2a
Placebo
19HL 3PI
19H 3PI
19H MD
wt 19
CRIa MD
WRSV
Live RSV
0
2
4
6
8
10
12
Anti-RSV-F Titre [Log2(titre/100)]

FIG. 2B.

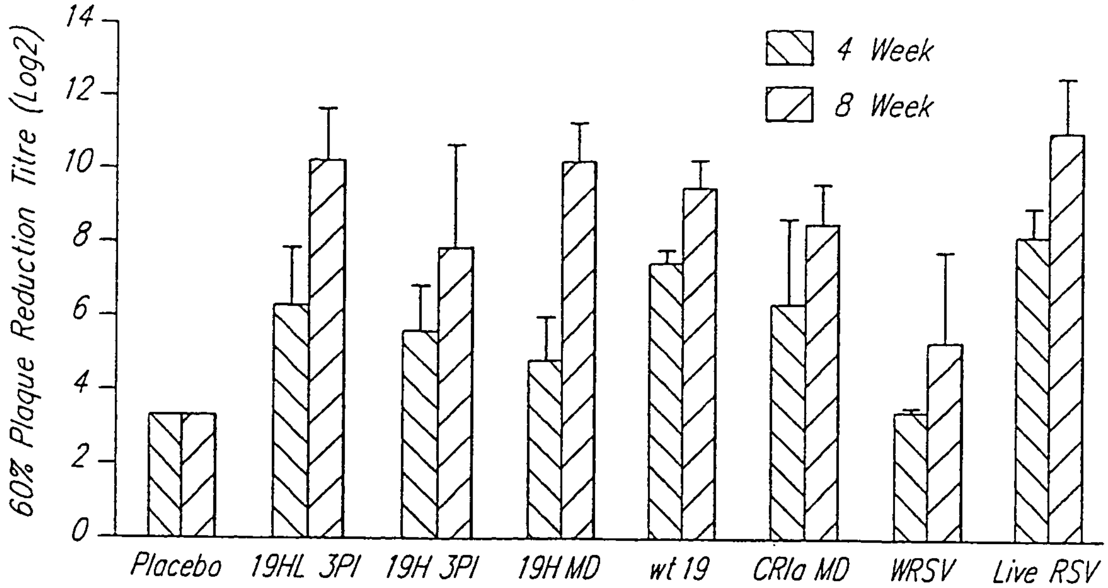

4 Week
8 Week
60% Plaque Reduction Titre (Log2)
0
2
4
6
8
10
12
14
Placebo
19HL 3PI
19H 3PI
19H MD
wt 19
CRIa MD
WRSV
Live RSV

FIG. 3.

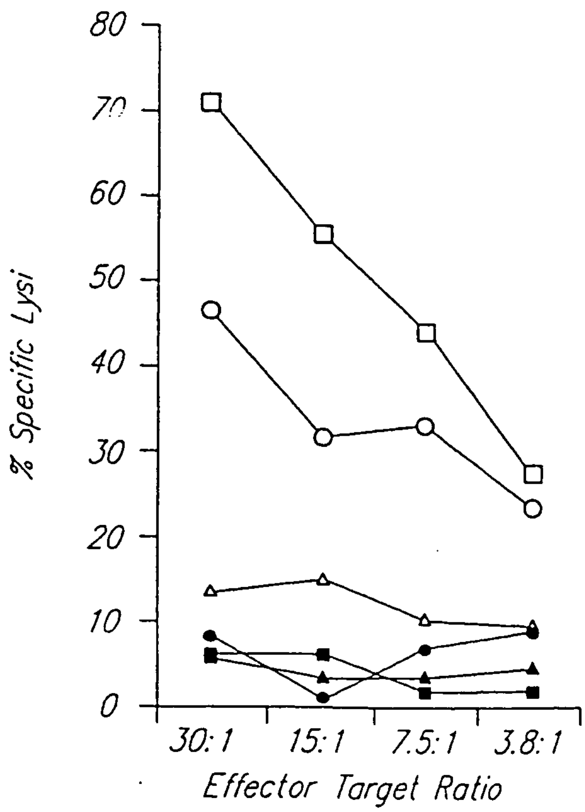

% Specific Lysi
0
10
20
30
40
50
60
70
80
30:1
15:1
7.5:1
3.8:1
Effector Target Ratio

FIG. 4.

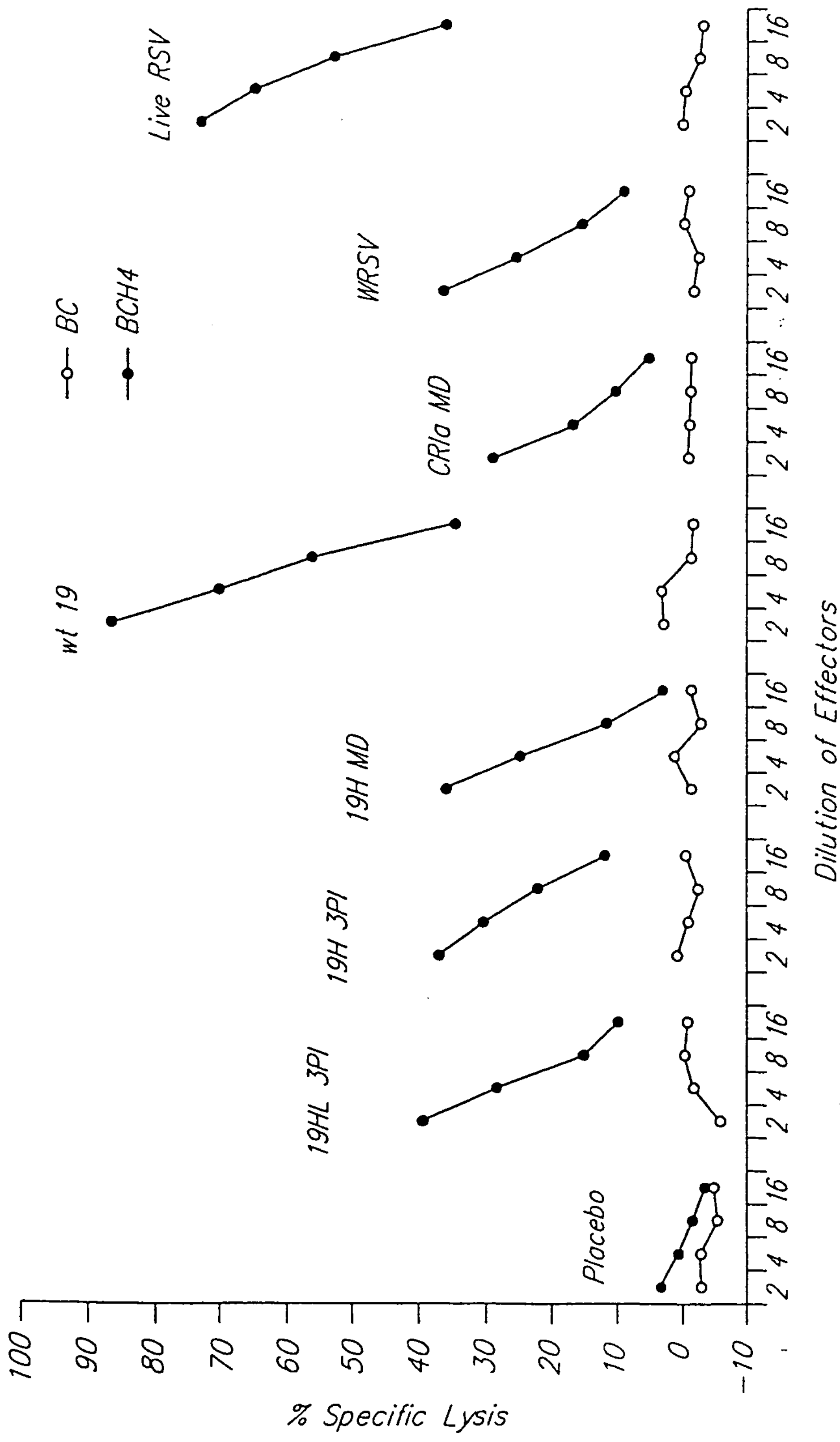

BC
BCH4
Live RSV
WRSV
CRla MD
wt 19
19H MD
19H 3PI
19HL 3PI
Placebo
2 4 8 16
Dilution of Effectors
100 90 80 70 60 50 40 30 20 10 0 −10
% Specific Lysis

FIG. 5.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/06636

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 39/155
US CL : 424/89
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/89

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, MEDLINE, WPIDS, CAPLUS
search terms: Respiratory syncytial virus, RSV, parainfluenza, vaccine, attenuat?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RANDOLPH et al. Attenuated temperature-sensitive respiratory syncytial virus mutants generated by cold adaptation. Virus Research. 1994, Vol. 33, pages 241-259, see the entire document.	1-22
Y	CROWE, JR. et al. Satisfactorily attenuated and protective mutants derived from a partially attenuated cold-passaged respiratory syncytial virus mutant by introduction of additional attenuating mutations during chemical mutagenesis. Vaccine. 1994, Vol. 12, No. 8, pages 691-699, see the entire document.	1-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
28 MAY 1998	30 JUL 1998

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	ALI R. SALIMI
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/06636

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CROWE, JR. et al. A comparison in chimpanzees of the immunogenicity and efficacy of live attenuated respiratory syncytial virus (RSV) temperature-sensitive mutant vaccines and vaccinia virus recombinants that express the surface glycoproteins of RSV. Vaccine. 1993, Vol. 11, No. 14, pages 1395-1404, see the entire document.	1-22
A	MURPHY et al. An update on approaches to the development of respiratory syncytial virus (RSV) and parainfluenza virus type 3 (PIV3) vaccines. Virus Research. 1994, Vol. 32, pages 13-36.	1-22
A	WRIGHT et al. Administration of a Highly Attenuated, Live Respiratory Syncytial Virus Vaccine to Adults and Children. Infection and Immunity. July 1982, Vol. 37, No. 1, pages 397-400.	1-22
A	MAASSAB et al. Development and characterization of cold-adapted viruses for use as live vaccines. Vaccine. December 1985, Vol. 3, No. 4, pages 355-369.	1-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/06636

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claim(s) 1-4 and 7, drawn to specific attenuated respiratory syncytial virus (RSV), and the first use (vaccine). (first product, and the first use).

Group II, claim(s) 5 and 6, drawn to method of determining the presence of antibodies against the RSV. (second use of the first product).

Group III, claim(s) 8-10, drawn to a composition comprising attenuated RSV, and a vaccine. (second product, and the use of the product).

Group IV, claim(s) 11 and 12, drawn to various mutants of RSV. (third product).

Group V, claim(s) 13-17 and 20-22, drawn to RSV selected from group wt19, and WRSV, and a vaccine. (forth product, and the first use of the product).

Group VI, claim(s) 18-19, drawn to method of determining antibodies of the forth product.(second use of the forth product).

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features (S.T.F) for the following reasons: The specific technical feature of Group I is the characteristics of the specific attenuated viruses listed in claim 1. Second product does not require special technical features of Group I because it is broader in scope than Group I and does not require the particular viruses required by Group 1. The third product, election of species is also required for explanation see below, does not require special technical features of Group I, because each virus in Group I has multiple mutations contributing to the characteristics of each strain. Since the special technical features of group I involves the combined effect of multiple mutations, the single mutations in isolation do not share the same special technical features. The forth product does not require the special technical features of Group I because it is drawn to different viruses.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: Explanation of election of species in the third product Group IV: Each mutation listed confers different structure and presumably different effect on antigenicity and virulence of the virus. Since effects of mutations are unpredictable, a mutation of one location does not teach or suggest mutation at a different location. Therefore, each location is a distinct species.

CORRECTED VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

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(74) Agents: SMITH, DeAnn, F. et al.; Harness, Dickey & Pierce, P.L.C., P.O. Box 828, Bloomfield Hills, MI 48303 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.
With an indication in relation to a deposited biological material furnished under Rule 13bis separately from the description.

(54) Title: ATTENUATED RESPIRATORY SYNCYTIAL VIRUS

(57) Abstract

Attenuated respiratory syncytial viruses (RSV) and in particular temperature sensitive RSV are provided. The viruses of the present invention may be used in pharmaceutical compositions such as vaccines. Methods of making and using such pharmaceutical compositions are also provided.

*(Referred to in PCT Gazette No. 25/1999, Section II)

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CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

ATTENUATED RESPIRATORY SYNCYTIAL VIRUS

RELATED APPLICATIONS

The present application is a continuation-in-part of PCT International Application No. PCT/US97/05588 (designating the United States), filed April 3, 1997, which claims priority under 35 U.S.C. §119(e) from U.S. Serial No. 60/014,848 , filed April 4, 1996. Both applications are hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention relates generally to attenuated respiratory syncytial viruses and, more particularly, to live attenuated respiratory syncytial virus vaccines and methods of protecting against disease caused by infection with respiratory syncytial virus.

BACKGROUND OF THE INVENTION

Respiratory syncytial virus (RSV), a member of the paramyxoviridae family, is the leading cause of viral pneumonia and bronchitis in infants and young children worldwide, and is a major cause of fatal respiratory tract disease. Serious disease is most prevalent in infants 6 weeks to 6 months of age and in children with certain underlying illnesses (*e.g.,* immunodeficiencies, congenital heart disease and bronchopulmonary dysplasia). Virtually all children are infected by two years of age. Most infections are symptomatic and are generally confined to mild upper respiratory tract disease. A decrease in severity of disease is associated with two or more prior infections and, in some studies, with high levels of serum antibody, suggesting that protective immunity to RSV disease will accumulate following repeated infections (Lamprecht, C.L. et al., *J. Inf. Dis.* 134:211-217 (1976); Henderson, F.W. et al., *N. Eng. J. Med.* 300:530-534 (1979); Glezen, W.P. et al., *J. Ped.* 98:706-715 (1981); Glezen, W.P. et al., *Am. J. Dis. Child.* 140:543-546 (1986); Kasel, J.A. et al., *Vir. Immunol.* 1:199-205 (1987/88); Hall, C.B. et al., *J. Inf. Dis.* 163:693-698 (1991)).

Two major subgroups of RSV have been identified, A and B, as well as antigenic variants within each subgroup (Anderson, L.J. et al., *J. Inf. Dis.* 151:626-633 (1985); Mufson, M.A. et al., *J. Gen. Virol.* 66:2111-2124 (1985)). Multiple variants of each subgroup have been found to co-circulate in epidemics which occur annually during late fall, winter, and spring months (Anderson, L.J. et al., *J. Inf. Dis.* 163:687-692 (1991)). There is evidence that children infected with one of the two major RSV subgroups may be protected against reinfection by the homologous subgroup (Mufson, M.A. et al., *J. Clin. Microbiol.* 26:1595-1597 (1987)). This, along with evidence that protective immunity will accumulate following repeated infections,

suggests that it is feasible to develop an RSV vaccination regiment for infants and young children which would provide sufficient immunity to protect against disease and death.

A previous attempt to vaccinate young children against RSV employed a parenterally administered formalin-inactivated RSV vaccine. Unfortunately, administration of this vaccine in several field trials was shown to be associated with the development of a significantly exacerbated illness following subsequent natural infection with RSV (Kapikian, A.Z. et al., *Am. J. Epidemiol.* 89:405-421 (1968); Kim, H.W. et al., *Am. J. Epidemiol.* 89:422-434 (1969); Fulginiti, V.A. et al., *Am. J. Epidemiol.* 89:435-448 (1969); Chin, J. et al., *Am. J. Epidemiol.* 89:449-463 (1969)).

Following the lack of success with the formalin-inactivated RSV vaccine, emphasis was placed on the development of live attenuated vaccines. For example, cold adaptation, a process by which virus is adapted to grow at temperatures colder than those at which it normally optimally grows, has been used to develop temperature sensitive, attenuated RSV mutants for consideration as vaccines (Maassab, H.F. et al., *Vaccine* 3:355-369 (1985)). Unlike chemical mutagenesis in which the genetic lesions are usually single, this method generally results in the accumulation of multiple genetic lesions. These multiple lesions would help to confer phenotypic stability by reducing the probability that reversion of any one lesion will result in reversion to virulence. Stepwise cold adaptation, wherein the virus is passaged multiple times at progressively lower temperatures, has been used to successfully develop several influenza vaccine candidates currently in clinical trials (Maassab, H.F. et al., *Viral Vaccines* Wiley-Liss, Inc. (1990); Obrosova-Serova, N.P. et al., *Vaccine* 8:57-60 (1990); Edwards, K.M. et al., *J. Inf. Dis.* 163:740-745 (1991)). These mutants, which bear attenuating mutations in at least four different genes, appear to be attenuated, immunogenic, and phenotypically stable.

RSV was cold-adapted to 25-26°C in several laboratories in the mid-1960's, but was found to be under-attenuated in vaccine trials (Kim, H.W. et al., *Pediatrics* 48:745-755 (1971); Maassab, H.F. et al., *Vaccine* 3:355-369 (1985)). However, it is of note that administration of these live RSV vaccine candidates was never associated with disease enhancement following natural infection.

Live attenuated vaccines offer several advantages over inactivated vaccines. These include the possible use of a single dose and administration by the natural route of infection *i.e.*, intranasally. In addition, live attenuated vaccines stimulate a wide range of immune responses, including local and serum antibody responses and

cellular immunity. Furthermore, these vaccines are cost-effective and can be rapidly produced and updated in the event of antigenic changes.

It would thus be desirable to provide avirulent (attenuated), immunoprotective and genetically-stable live attenuated RSV strains. It would further be desirable to provide a vaccine comprising such attenuated strains. It would further be desirable to provide methods of making and using said RSV vaccine to protect against disease caused by infection with RSV.

SUMMARY OF THE INVENTION

Attenuated RSV strains which exhibit the cold-adapted (*ca*) and/or temperature sensitive (*ts*) phenotype are provided. Samples of viruses as embodiments of the present invention have been deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20582, under the terms of the Budapest Treaty, and have been accorded the following ATCC designation numbers:

Table 1

Virus	Phenotype	Description	ATCC Designation No.	Date of Deposit
CRIa	temperature sensitive/cold-adapted	RSV, Ia-CRSV-5 CL 15 MRC27	VR-2511	September 20, 1995
Ca19S	temperature sensitive/cold-adapted	RSV, Line 19 MRC5-15-25° st-33°	VR-2512	September 20, 1995
19H	temperature sensitive	RSV, Line 19 MRC5-60-35°	VR-2513	September 20, 1995
Ca48V	temperature sensitive/cold-adapted	RSV, Line 48 MRC5-14-25° st MRC1-33° VERO 10-25° VERO 1-33°	VR-2514	September 20, 1995
Ca19V	temperature sensitive/cold-adapted	RSV, Line 19 MRC5-10-25° VERO 16-25° VERO 6-20° VERO 3-33°	VR-2515	September 20, 1995
CaBCV	temperature sensitive/cold-adapted	RSV, CRSV-BC5 CL-17 MRC30	VR-2516	September 20, 1995
CaBCL	temperature sensitive/cold-adapted	RSV, CRSV-BC13 MRC19-25° MRC1-33°	VR-2517	September 20, 1995
19H 4MD	temperature sensitive	RSV, Line 19 MRC5 92-35°C, Clone 4-1, MRC5, P-103-33° Purified by minimal limited dilution (MLD)	VR-2567	April 2, 1997
19H 3PI	temperature sensitive/cold-adapted	RSV, Line 19 MRC 70-35°C, Vero 3-35°C, MRC5 2-35°C Clone 2-35°C (3PI) Plaque purified	VR-2564	April 2, 1997

Table 1 (con't.)

Virus	Phenotype	Description	ATCC Designation No.	Date of Deposit
19H 5MD	temperature sensitive	RSV, Line 19 MRC5, 92-35°C Clone 5-1 Purified by MLD	VR-2565	April 2, 1997
CRIa MD	temperature sensitive/cold-adapted	RSV, la-CRSV-5 MRC-38-25°C, MRC1-33°C Purified by MLD	VR-2566	April 2, 1997
19HL 3PI	temperature sensitive/cold-adapted	RSV, Line 19 MRC5 72-35°C VERO 3-35°C Large Clone 6-35°C, (3PI) Plaque Purified	VR-2572	April 23, 1997
wt 19		Progenitor wt RSV, line 19 MRC5 1-33°C, VERO 2-35°C, MRC5 1-35°C Purified by MLD	VR-2570	April 23, 1997
WRSV		Progenitor wt RSV, WI 38-3 MRC5 9-35°C, MRC5 1-35°C, Purified by MLD	VR-2571	April 23, 1997

The deposit of the viruses will be maintained in the ATCC depository, which is a public depository, for a period of 30 years, or 5 years after the most recent request, or for the effective life of a patent, whichever is longer, and will be replaced if the deposit becomes depleted or nonviable during that period. Samples of the deposited strains will become available to the public and all restrictions imposed on access to the deposits will be removed upon grant of a patent on this application.

The present invention also provides methods for immunizing a subject against disease caused by infection by RSV comprising administering to the subject an immunoeffective amount of an attenuated RSV and in particular, cold-adapted and/or temperature sensitive RSV. Methods of making and using such attenuated RSV in a pharmaceutical composition *e.g.*, a vaccine, are also provided.

Additional objects, advantages, and features of the present invention will become apparent from the following description, drawings and appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The various advantages of the present invention will become apparent to one skilled in the art by reading the following specification and subjoined claims and by referencing the following drawings in which:

Figure 1A is a graph showing the total anti-F IgG response of mice immunized with an immunogenic composition of an aspect of the present invention;

Figure 1B is a graph showing the anti-F IgG_1 response of mice immunized with an immunogenic composition of an aspect of the present invention;

Figure 1C is a graph showing the anti-F IgG_{2a} response of mice immunized with an immunogenic composition of an aspect of the present invention;

Figure 2A is a graph showing the anti-RSV-F antibody titers (after 4 weeks) of mice immunized with an immunogenic composition of an aspect of the present invention;

Figure 2B is a graph showing the anti-RSV-F antibody titers (after 8 weeks) of mice immunized with an immunogenic composition of an aspect of the present invention;

Figure 3 is a graph showing RSV specific neutralizing antibody titers (after 4 and 8 weeks) of mice immunized with an immunogenic composition of an aspect of the present invention;

Figure 4 is a graph showing cytotoxic T cell (CTL) activity of mice immunized with an immunogenic composition of an aspect of the present invention; and

Figure 5 is a graph showing CTL activity of mice immunized with immunogenic compositions of aspects of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

Attenuated RSV including cold-adapted and/or temperature sensitive RSV, as well as progenitor viruses, are provided and have been deposited with the ATCC and are described in detail herein. As used herein, the term "cold-adapted" means a virus that has been attenuated by propagation at lower than optimal growth temperatures and the term "temperature sensitive" means that replication of the virus is impeded as temperature is elevated.

The lines of the present invention have been successfully attenuated using three different approaches: adaption to suboptimal temperature by direct and stepwise passage; high passages at 35°C; and, adaption to a heterologous host (*i.e.*, host-range restricted). Four of the deposited lines have also been plaque purified (19HL 3PI, 19H 3PI, 19H MD and CRla MD). The attenuated RSV of the present invention are genetically-stable, immunogenic and protective, and avirulent, and are thus particularly useful in the formulation of live, attenuated RSV vaccines which are capable of eliciting a protective immune response without causing unacceptable symptoms of severe respiratory disease. The immune response which is achieved in the subject by the method of an embodiment of the present invention preferably includes the production of virus specific neutralizing antibodies and the virus specific cytotoxic T-cell responses. The invention is therefore particularly effective to provide protection against respiratory tract diseases caused by RSV.

Methods of attenuating RSV, for example, attenuating the deposited progenitor viruses, as well as methods of making and using attenuated RSV vaccines, are also provided by the present invention, including the preparation of pharmaceutical compositions.

Nucleic acid molecules encoding the attenuated RSV are also within the scope of the present invention. These nucleic acids may be DNA molecules, cDNA molecules or RNA molecules *e.g.*, antisense RNA. The present invention further includes nucleic acid molecules which differ from that of the nucleic acid molecules which encode the RSV of the present invention, but which produce the same cold-adapted and temperature sensitive phenotypic effect. These altered, phenotypically equivalent nucleic acid molecules are referred to as "equivalent nucleic acids." The present invention also encompasses nucleic acid molecules characterized by changes

in non-coding regions that do not alter the phenotype of the polypeptide produced therefrom, when compared to the nucleic acid molecules of the RSV described herein.

Also encompassed by the present invention are the nucleic acid molecules comprising noncoding sequences of the RSV of the present invention. These non-coding regions are to include 5′ noncoding regions, 3′ noncoding regions, intergenic sequences, and other noncoding regions of the viral genome. These include, but are not limited to, transcriptional, translational and other regulatory regions. These nucleic acid molecules also may be DNA molecules, cDNA molecules or RNA molecules.

Nucleic acid molecules which hybridize under stringent conditions to the nucleic acid molecules described herein are also within the scope of the present invention. As will be appreciated by those skilled in the art, multiple factors are considered in determining the stringency of hybridization including species of nucleic acid, length of nucleic acid probe, T_m (melting temperature), temperature of hybridization and washes, salt concentration in the hybridization and wash buffers, aqueous or formamide hybridization buffer, and length of time for hybridization and for washes. An example of stringent conditions are DNA-DNA hybridization with a probe greater than 200 nucleotides in 5 x SSC, at 65°C in aqueous solution or 42°C in formamide, followed by washing with 0.1 x SSC, at 65°C in aqueous solution. (Other experimental conditions for controlling stringency are described in Maniatis, T. et al., *Molecular Cloning: a Laboratory Manual*, Cold Springs Harbor Laboratory, Cold Springs, N.Y. (1982) at pages 387-389 and Sambrook, J. et al., *Molecular Cloning: a Laboratory Manual*, Second Edition, Volume 2, Cold Springs Harbor Laboratory, Cold Springs, N.Y., at pages 8.46-8.47 (1989)).

The nucleic acid molecules of the present invention may be operatively-linked to a promoter of RNA transcription, as well as other regulatory sequences. As used herein, the term "operatively-linked" means positioned in such a manner that the promoter and other regulatory sequences will direct the transcription off of the nucleic acid molecule. An example of a promoter is the T7 promoter. Vectors which contain both a promoter and a cloning site to which an inserted piece of nucleic acid is operatively-linked to the promoter, are well known in the art. Preferably, these vectors are capable of transcribing nucleic acid *in vitro* and *in vivo*.

Purified polypeptides isolated from the RSV described herein or from cells infected with these same virus, are also encompassed by the present invention. The

polypeptides (or fragments thereof) may be of varying length, and preferably will be capable of exhibiting immunological activity.

Methods for producing polypeptides of the present invention are also within the scope of the present invention. In one embodiment, RSV polypeptides can be isolated in substantially pure form from RSV or cultures of cells infected with RSV. In an alternative embodiment, RSV polypeptides can be isolated from a recombinant system or are vector-engineered to produce these polypeptides. In yet another embodiment, RSV polypeptides can be chemically synthesized by methods well known to those of skill in the art.

All derived RSV strains including the deposited attenuated RSV derived from the progenitor viruses, are also encompassed by the present invention, including, without limitation, those attenuated by cold adaptation (including both direct and stepwise passage), high *in vitro* passage, host-range restriction and chemical or genetic modification *e.g.*, site-directed mutagenesis.

Although the deposited RSV of the present invention are subgroup A virus, it will be appreciated by those skilled in the art that subgroup B virus can be produced by biologically cloning wild-type subgroup B virus in an acceptable cell substrate using methods known in the art. The subgroup B virus may then be attenuated as described herein.

Pharmaceutical compositions comprising any of the RSV described herein or polypeptides, either alone or in combination, and a pharmaceutically acceptable carrier, are also provided by the present invention. As used herein, the phrase "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as physiologically balanced culture medium, phosphate buffered saline solution, water, and emulsions, such as an oil/water emulsion, various types of wetting agents and protein stabilizers. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Formulations include those suitable for oral, nasal, topical (including transdermal, buccal and sublingual), parenteral (including subcutaneous) and pulmonary administration. The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any method known in the art.

In one embodiment of the present invention, the pharmaceutical composition is intended for use as a vaccine. In such embodiment, a virus may be mixed with cryoprotective additives or stabilizers such as proteins (*e.g.*, albumin, gelatin), sugars

(*e.g.*, sucrose, lactose, sorbitol), amino acids (*e.g.*, sodium glutamate), saline, or other protective agents. This mixture may then be desiccated or lyophilized for transport and storage, and reconstituted prior to administration. Lyophilized virus will typically be maintained at about 4°C and when ready for use, reconstituted in a stabilizing solution, with or without adjuvant. In yet another embodiment of the present invention, the virus may be inactivated and may be mixed with an adjuvant, saline and a detergent such as phosphate Tween buffer. For further methods of vaccine preparation, see Duffy, J.I., *Vaccine Preparation Techniques*, Noyes Data Corporation, (1980).

Immunogenicity can be significantly improved if the virus is co-administered with an immunostimulatory agent or adjuvant. Adjuvants enhance immunogenicity but are not necessarily immunogenic themselves. Immunostimulatory agents or adjuvants have been used for many years to improve the host immune responses to, for example, vaccines.

Suitable adjuvants are well known to those skilled in the art and include, without limitation, aluminum phosphate, aluminum hydroxide, QS21, Quil A, derivatives and components thereof, calcium phosphate, calcium hydroxide, zinc hydroxide, a glycolipid analog, an octodecyl ester of an amino acid, a muramyl dipeptide, polyphosphazene, a lipoprotein, ISCOM matrix, DC-Chol, DDA, and other adjuvants and bacterial toxins, components and derivatives thereof.

Pharmaceutical compositions comprising any of the attenuated RSV of the present invention are useful to immunize a subject against disease caused by RSV infection. Thus, this invention further provides methods of immunizing a subject against disease caused by RSV infection, comprising administering to the subject an immunoeffective amount of a pharmaceutical composition of the invention. This subject may be an animal, for example a mammal, such as a primate or preferably a human.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, immunogenic and protective. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the immune system of the individual to synthesize antibodies, and, if needed, to produce a cell-mediated immune response. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and may be monitored on a patient-by-patient basis. However, suitable dosage ranges are readily determinable by one skilled in the art

and generally range from about 10^2 to about 10^9 plaque forming units (PFU) or more of virus per patient, more commonly, from about 10^4 to about 10^5 PFU of virus per patient. The dosage may also depend, without limitation, on the route of administration, the patient's state of health and weight, and the nature of the formulation.

It will be appreciated that administration of the vaccines of the present invention will be by procedures well established in the pharmaceutical arts, such as intranasally, parenterally, intravenously, orally, or topically applied to any mucosal surface such as intranasal, oral, eye or rectal surface. Moreover, as described in more detail in Specific Example 3E., more than one route of administration may be employed either simultaneously or sequentially (*e.g.*, boosting). In a preferred embodiment of the present invention, live, attenuated viral vaccines are administered intranasally, orally, parenterally or applied to any mucosal surface (nasal, oral, eye, rectal). Inactivated whole virus vaccine is preferably administered parenterally or to any mucosal surface.

Upon inoculation with an attenuated RSV pharmaceutical composition as described herein, the immune system of the host responds to the vaccine by producing antibodies, both secretory and serum, specific for RSV proteins. As a result of the vaccination, the host becomes at least partially or completely immune to RSV infection, or resistant to developing moderate or severe RSV infection, particularly of the lower respiratory tract.

It will be appreciated that the attenuated RSV of the present invention can be combined with viruses of other subgroups or strains to achieve protection against multiple strains of RSV. Typically the viruses will be in an admixture and administered simultaneously, but may also be administered separately. Due to the phenomenon of cross-protection among certain strains of RSV, immunization with one strain may protect against several different strains of the same or different subgroup.

In some instances it may be desirable to combine the attenuated RSV vaccines of the present invention with vaccines which induce protective responses to other agents, particularly other childhood viruses. For example, the vaccine compositions of the present invention can be administered simultaneously, separately or sequentially with other vaccines such as parainfluenza virus vaccine, as described in Clements, et al., *J. Clin. Microbiol.* 29:1175-1185 (1991). Moreover, a multivalent preparation may be employed comprising for example, the attenuated RSV of the

present invention (including subgroups A and B), parainfluenza virus type 1, 2 and 3 and influenza virus types A and B.

It will also be appreciated that single or multiple administrations of the vaccine compositions of the present invention may be carried out. In neonates and infants, multiple administration may be required to elicit sufficient levels of immunity. Administration should begin within the first month of life, and continue at intervals throughout childhood, such as at two months, six months, one year and two years, as necessary to maintain sufficient levels of protection against wild-type RSV disease. Similarly, adults who are particularly susceptible to repeated or serious RSV infection, such as, for example, health care workers, day care workers, elderly and individuals with compromised cardiopulmonary function, may require multiple immunizations to establish and/or maintain protective immune responses. Levels of induced immunity can be monitored by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to maintain desired levels of protection.

Those skilled in the art will further appreciate that the viruses of the present invention may be used in diagnostic applications. For example, a method of determining the presence of antibodies specifically reactive with an RSV of the present invention is provided. Such a method comprises the steps of contacting a sample with the RSV to produce complexes comprising the virus and any antibodies present in the sample specifically reactive therewith, and determining production of the complexes. A similar method of determining the presence of RSV is provided wherein the sample is contacted with an antibody specifically reactive with an RSV to produce complexes comprising the antibody and the virus present in the sample that is specifically reactive with the antibody, and determining production of the complexes.

The virus of the present invention are characterized by a level of attenuation such that they do not produce RSV disease in a host immunized therewith, evoke a protective immune response and do not lead to immunopotentiation or exacerbated disease. They lack transmissibility, are genetically stable and exhibit cold-adapted and temperature sensitive markers. They are immunogenically protective and induce protective levels of humoral and cell mediated immunity. In particular, a balanced anti-RSV F IgG1/IgG2a response is seen in hosts immunized with attenuated viruses of the present invention. They can be administered by the natural route *i.e.*, intranasally. The RSV of the present invention may be tested in *in vitro* and *in vivo*

models to demonstrate these characteristics. A variety of animal models have been described and are summarized in Meignier et al., eds., *Animal Models of Respiratory Syncytial Virus Infection*, Merieux Foundation Publication (1991). A cotton rat model of RSV infection is described in U.S. 4,800,078 and Prince et al., *Virus Res.* 3:193-206 (1985), and is believed to be predictive of attenuation and efficacy in humans. A primate model of RSV infection using a chimpanzee is also useful in examining attenuation and protection and is described in detail in Richardson et al., *J. Med. Virol.* 3:91-100 (1978) and Wright et al., *Infect. Immun.* 37:397-400 (1982).

SPECIFIC EXAMPLES

The following Specific Examples illustrate practice of the invention. These examples are for illustrative purposes only and are not intended in any way to limit the scope of the claimed invention.

Specific Example 1 describes the production and characterization of the virus of the present invention including the passage status and procedures for developing the strains.

Specific Example 2 describes temperature sensitivity studies, wherein the deposited strains were found to have the *ts* phenotype (see Tables 2 and 3).

Specific Example 3 describes immunogenicity studies wherein mice were immunized by administering an RSV strain of the present invention. As described in Specific Examples 3A.-3D., mice were immunized and sera examined four weeks after boosting for anti-F, total IgG antibodies, IgG1 and IgG2a antibodies. The results are shown in Figures 1A-3 and Tables 4-7 and show that the intranasal immunization with the attenuated RSV produces a substantial anti-F antibody response. In particular, a balanced anti-RSV F IgG1/IgG2a response demonstrating the induction of both Th-1 and Th-2 type responses was achieved. The generation of IgG2A antibodies in the murine model is indicative of a Th1-type immune response. The level of virus-neutralizing antibodies was also determined, by plaque reduction assays.

In Specific Example 3E., a study was performed to evaluate the effect of boosting by a route of administration that differs from the initial inoculation route of administration. In particular, mice were inoculated intranasally and boosted in the footpad and intramuscularly. Neutralization titer data is set forth in Table 8.

As described in Specific Examples 3F. and 3G., the generation of RSV-specific cytotoxic T cells (CTL) following immunization was determined and the results shown in Figures 4 and 5. Immunizing animals with the attenuated RSV of the present invention induced significant levels of CTL activity. In addition, as shown in Table 9,

mice immunized with the plaque purified viruses of the present invention were protected against live virus challenge.

Specific Example 4 describes sequence analysis of the F gene of several RSV lines. Specific Example 4A. sets forth the comparison of the sequences for wild type RSV (referred to herein as WRSV) and two attenuated lines, Ca19V and 19H (see Table 10). Specific Example 4B. illustrates the comparison of the sequences for the line 19 progenitor strain (referred to herein as wt 19) and the same two lines, lines Ca19V and 19H (see Table 11). The F genes of the two attenuated lines both differ from the WRSV as well as the wt 19, but have 66 nucleotides and 11 amino acids in common. With respect to the amino acid differences between the attenuated lines and the wt 19, none of the amino acid differences are shared by the two attenuated lines.

Specific Exarnple 5 describes the plaque purification of the attenuated RSV lines of the present invention. Table 12 illustrates the temperature sensitivity of the plaque purified lines.

Specific Example 6 describes RSV therapeutic protocols for administering the pharmaceutical compositions of the present invention to humans.

SPECIFIC EXAMPLE 1 - PRODUCTION AND CHARACTERIZATION OF VIRUS

A. The following sets forth the deposited RSV strains and their titers.

1. 19H
   in EMEM 5% FBS 5% Glycerol
   1.00×10^8 $TCID_{50}$ in MRC Tubes on Day 14
   1.85×10^5 Pfu's/ml in VERO cells
   ATCC Designation No. VR-2513

2. Ca19S
   in EMEM 5% FBS 5% Glycerol
   7.00×10^4 Pfu's/ml in VERO cells
   ATCC Designation No. VR-2512

3. Ca19V
   in 199 5% FBS 5% Glycerol
   1.00×10^7 Pfu's/ml in VERO cells
   ATCC Designation No. VR-2515

4. Ca48V
in 199 5% FBS 5% Glycerol
9.00×10^4 Pfu's/ml in VERO cells
ATCC Designation No. VR-2514

5. CaBCV
in 199 5% FBS 5% Glycerol
1.30×10^4 Pfu's/ml in VERO cells
ATCC Designation No. VR-2516

6. CRIa
in 100 5% FBS 5% Glycerol
6.00×10^3 Pfu's/ml in VERO cells
ATCC Designation No. VR-2511

7. CaBCL
in 199 5% FBS 5% Glycerol
1.70×10^4 in VERO cells
ATCC Designation No. VR-2517

8. 19H 4MD
in 5% Glycerol
3.2×10^5 $TCID_{50}$/ml in MRC5 cells
ATCC Designation No. VR-2567.

9. 19H 3PI
in 5% Glycerol
4×10^5 $TCID_{50}$/ml in MRC5 cells
ATCC Designation No. VR-2564.

10. 19H 5MD
in 5% Glycerol
3.2×10^5 $TCID_{50}$/ml in MRC5 cells
ATCC Designation No. VR-2565.

11. CRIa MD
in 5% Glycerol
4 x 10^5 $TCID_{50}$/ml in MRC5 cells
ATCC Designation No. VR-2566.

12. 19HL 3PI
in 5% Glycerol
2.0 x 10^6 $TCID_{50}$/ml in MRC5 cells
ATCC Designation No. VR-2572.

13. wt 19
in 5% Glycerol
3.0 x 10^5 $TCID_{50}$/ml in MRC5 cells
ATCC Designation No. VR-2570.

14. WRSV
in 5% Glycerol
2.0 x 10^5 $TCID_{50}$/ml in MRC5 cells
ATCC Designation No. VR-2571.

B. The following depicts the passage status of exemplary derivative RSV strains of the present invention.

1. Line 19 MRC5 72-35°
2. Line 19 MRC5 24-25° st 1-33°
3. Line 19 MRC5 10-25° VERO 16-25° 6-20° 3-33°
4. Line 48 MRC 14-25° st 1-33° VERO 10-25° 1-33°
5. CRSV-BC5 CL17 MRC 30-25°
6. la-CRSV-5 CL15 MRC 27-25°
7. CRSV-BC13 MRC 19-25° 1-33°
8. Line 19 MRC5 P-70-35°, VERO P-3-35°, MRC5, P-2-35° clone 2 (3PI) plaque purified
9. Line 19 MRC5, P-92-35°, clone 5-1, purified by minimal limited dilution (MLD)
10. la-CRSV-5, MRC5, P-38-25°, MRC5, P-1-33°, purified by MLD
11. Line 19 HP clone 4-1, MRC5, P-103-33°, purified by MLD

12. Line 19 MRC 70-35°C, Vero P-35°C, large clone 6-35° (3PI), plaque purified

C. The following are the procedures for developing the attenuated deposited RSV strains of the present invention (see Specific Example 5 for additional details regarding the plaque purification). CRIa, CRIa MD, CaBCL and CaBCV were derived from WRSV. 19H, Ca19S, Ca19V, 19H 4MD, 19H 3PI, 19H 5MD and 19HL 3PI were derived from wt 19.

1. 19H

MRC5 cells were purchased in tubes from Bio Whittaker Laboratories. Media was removed. 1.2 ml EMEM + 5% FBS was added to each tube. 0.3 ml virus was added to each of 4 tubes. Tubes were incubated at 35°C and observed for development of cytopathic effect (CPE). Tubes were frozen at -70°C. Virus was harvested and passed to fresh cells.

2. Ca19S

The same steps as in 1. above were performed, except that tubes were passed 10 times at 35°C; 10 times at 30°C; 15 times at 25°C, and 2 times at 33°C.

3. Ca19V

The first 10 passages in MRC5 cells were performed as described in 1. above except that tubes were incubated at 25°C. Media was removed from 3 confluent $25cm^2$ VERO flasks. Virus was diluted 1:5 in 1 x 199 + 5% FBS. 1 ml of virus was added to each of 2 flasks (1 flask is used as a control). Virus was adsorbed on a rocker at room temperature for 2 hours. 4 ml of 1 x 100 +5% FBS was added to each flask. Flasks were incubated at the appropriate temperature until 80% CPE was observed. Flasks were frozen at -70°C. Virus was harvested and passed to fresh cells. Virus was passed 16 times at 25°C; 6 times at 20°C; and, 2 times at 33°C.

4. a. Ca48V

The first 14 passages in MRC5 cells were performed as described in 2. above. Passage in VERO cells was performed as in 3. above. Virus was passed 10 times in VERO cells at 25°C and 1 time at 33°C.

5. CaBCV, CRIa and CaBCL

Same as 2. above except that 199 + 5% FBS was used.

An efficient plaque purification system was used (where indicated), which can evaluate individual plaques for temperature sensitivity. The following procedures were employed. Monolayers of Vero cells grown in 6- or 12-well plates were infected with 10-fold serial dilutions of virus in EMEM medium supplemented with 5% FBS and L glutamin. Each dilution was plated into three replicate wells. Virus was absorbed by incubation at 35°C on a rocker for 2 hours. The inoculum was removed and the cells were overlaid with 0.6% (W/V) Sea Kem ME agarose (final concentration) and 1xEMEM 3.5% FBS, L glutamine and gentamycin. The plates were allowed to solidify at room temperature and were incubated in CO_2 in parallel at 25°C, 33°C, 37°C and 39°C for 4 days. To clearly visualize developing plaques, a second overlay of agarose medium containing the same first overlay agarose medium with additional .01% (W/V) neutral red, was added on the fourth date after infection and plates were incubated in CO_2 at the appropriate temperature. Individual plaques were picked and were emulsified in 0.5 ml EMEM 5% FBS and either amplified or frozen at -70°C.

Each virus was evaluated by the time of plaque appearance, the plaque morphology, size and its characterization and titer. The growth of a given virus was expressed as (PFU/ml) for titration. The picked plaques were used to inoculate duplicate tubes containing Vero cell monolayers. One duplicate was incubated at 33°C and the other at 39°C up to 14 days. Cultures were checked for virus CPE. The tubes which demonstrated easily detectable CPE at 33°C, and no CPE at 39°C were selected for further plaque purification, titration and temperature sensitivity studies.

Conventional minimal limited dilution procedures, known to those skilled in the art, were followed where indicated.

SPECIFIC EXAMPLE 2 - TEMPERATURE SENSITIVITY STUDIES

To screen the cold-adapted and high passage virus for the presence of temperature sensitive (*ts*) variants, viruses were tested at 39°C, 37°C and 33°C by one of two methods of titration: plaque immunoassay or $TCID_{50}$.

Results

Line 19H is *ts* when assayed in both MRC cells and under agarose in Vero cells. Line Ca19S has a 5 log reduction in growth at 39°C versus that at 33°C. Line CRIa is also *ts* in MRC cells. WRSV grows as well at 39°C as it does at 33°C.

Lines Ca19V and Ca48V are both *ts* when assayed in Vero cells using the second antibody technique and under agarose. Line Ca19V has a 5 log reduction in

growth at 39°C versus that at 33°C and line Ca48V has a 3 log reduction in growth at the non-permissive temperature.

The following tables further set forth the results of the temperature sensitivity study.

Table 2

$TCID_{50}$ in MRC Cells on Day 14

Virus Lines	33°C	37°C	38°C	39°C
19H	1.00×10^8			3.16×10^5
19H	4.68×10^6	2.15×10^5		4.68×10^4
Ca19S	3.16×10^7		3.16×10^3	3.16×10^2
CRla	4.68×10^3		4.68×10^2	3.16×10^1
WRSV	3.16×10^4		3.16×10^4	1.00×10^4

Table 3

Pfu's in Vero Cells on Day 7

Virus Lines	33°C	37°C	38°C	39°C
Ca19V	3.16×10^{10}		5.85×10^9	2.54×10^5 pinpoint plaques
Ca48V	2.50×10^7	8.00×10^6		6.00×10^4 pinpoint plaques

Materials and Methods

$TCID_{50}$ in MRC5 Cells. Virus to be titrated was diluted 10^{-1} in EMEM + 5% FBS. Confluent MRC5 tubes were used, for each dilution and for each temperature (total of 96 tubes for 3 temperatures). 1 ml of a viral dilution was added to each tube. Tubes were incubated at 33°C, 37°C or 38°C, and 39°C. Tubes were read daily to day 14 for CPE. $TCID_{50}$ was calculated using the method of Reed and Muench.

Plaque Immunoassay - Pfu's in HEP2 Cells or Vero Cells. HEP2 Cells (or VERO cells) were grown in a 12 well microtiter plate until semi-confluent and media was removed. Virus was diluted in 1 x 199 + 5% FBS 10^{-1} to 10^{-7}. Cells were inoculated in triplicate, 0.5 ml/well and allowed to adsorb at 35°C for 2 hours. Inoculum was then removed. The cells were overlaid with 2 ml of the 1:1 mixture of

2 x EMEM with 6% FBS and the 4% Methyl Cellulose (4 gm Methyl Cellulose and 100 ml Type I Deionized Water, autoclaved to sterilize; final concentration 2% Methyl Cellulose) and incubated at 35°C with 5% CO_2 for 7 days. Overlay media was then discarded. The cells were fixed with cold 80% methanol at -70°C for 1 hour. The methanol was then removed and the plates were frozen at -70°C. Plates were allowed to thaw at room temperature. 1 ml of 5% Blotto media (25 gm Milk and 500 ml PBS) was added to each well and the wells were incubated at 35°C for 30 minutes. 5% Blotto media was then removed and 1 ml of 5% Blotto media with 1/100 dilution of anti-RSV antibody was added. Incubation at 35°C for 30 minutes took place. After incubation, 5% Blotto media with antibody was removed and cells were washed with 5% Blotto media. 1 ml of 5% Blotto media with 1/100 dilution of conjugate antibody was then added and incubation took place at 35°C for 30 minutes. 5% Blotto media with conjugate antibody was removed and cells were washed with PBS. 1 ml of 1:1 mix of peroxide solution substrate (4 chloro-1 napthol) + H_2O_2 was added and incubation took place at room temperature for 1-5 minutes. During this period color development was watched carefully. Cells were then washed with PBS. Plaques were counted and Pfu's recorded.

SPECIFIC EXAMPLE 3 - IMMUNOGENICITY STUDIES

A. Immunogenicity of RSV Lines (Study 1)

A study was performed to determine the immunogenicity of line 19H. Pathogen-free BALB/c mice (approximately 8 weeks old) were immunized intranasally with either 1.6 X10^6 $TCID_{50}$ of 19H, 2.5 X 10^5 pfu of A2 mouse adapted virus (designated live virus), or 5% glycerol (designated placebo). Animals were bled 4 weeks after the primary inoculation and boosted at 4 weeks with an equivalent dose of the vaccine formulation. Serum samples were also taken 4 weeks after the booster dose. Anti-F antibody titer was determined as follows: immunoaffinity purified RSV-F antigen was coated on wells of Nunc-immuno Maxi Sorp flat bottom microtiter plates, by incubating antigen overnight at room temperature in 0.05M Carbonate - Bicarbonate buffer, pH 9.6. Wells were blocked for non-specific binding by adding 0.1% BSA in PBS for 30 min. at room temperature, followed by two washes in a washing buffer of 0.1% BSA in PBS + 0.1% Tween 20. Mice sera was diluted in two or four-fold serial dilutions, and added to wells. After 1 hour incubation at room temperature, plates were washed five times with washing buffer, and horseradish peroxidase labeled conjugate was added at the appropriate optimal dilution in washing buffer. The total IgG assay used $F(ab')_2$, goat anti-mouse IgG (H+L specific)-HRP

from Jackson Immuno Research Laboratory Inc., Baltimore. The IgG 1 assay used sheep anti-mouse IgG1-HRP from Serotec. The IgG 2a and IgA assays used goat anti-mouse IgG 2a-HRP and goat-anti-mouse IgA-HRP respectively, from Caltag Laboratories, San Francisco. Following 1 hour incubation at room temperature, the plates were washed five times with washing buffer, and a substrate hydrogen peroxide in the presence of tetramethylbenzidine was added. The color reaction was stopped by adding 2M sulfuric acid. The color was read in a Multiscan Titertek plate reader at an optical density (OD) of 450nm. The titre was taken as the reciprocal of the last dilution at which the OD was approximately double. This OD must be greater than the negative control of the assay at the starting dilution. The pre-immune serum of each animal was used as the negative control.

Line 19H elicited levels of anti-F IgG antibodies that were equivalent to those induced by the A2 mouse adapted virus (see Figures 1A, 1B and 1C). Sera from animals that received two doses of line 19H had a balanced anti-RSV F Ig_1/IgG_{2a} response. As outlined in the table below, the sera of animals that were immunized with two doses of 19H had RSV-specific neutralizing antibodies that were comparable to those obtained following inoculation with live mouse adapted RSV. Thus, line 19H was immunogenic in the mouse model.

Table 4

Serum antibody response of BALB/c mice immunized with *ts* mutant

Formulation	Neutralization titer[a,b] ($\log_2 \pm$ s.d.)	
	4 Week Bleed	8 Week Bleed
19H	8.2 ± 0.9	9.5 ± 1.2
Live RSV (mouse-adapted A2 virus)	8.5 ± 0.8	10.6 ± 0.7
Placebo	<3.3 ± 0.0	<3.3 ± 0.0

[a]Neutralization titer determined by complement-enhanced 60% plaque-reduction assay
[b]Each value represents the reciprocal mean titer of at least 6 animals

B. Immunogenicity of RSV Lines (Study 2)

A second study was performed to determine the immunogenicity of the following RSV lines: WRSV (Vero $35°_1$, titer 2.00×10^6 Pfu's/ml in Vero cells), CRla, CaBCV, 19H, Ca19V and Ca48V. Balb/c/AnNTacfBR three week old male mice from Taconic, 272 Hoover Avenue, Germantown, NY 12526, were used. Mice were

anesthetized IP with 200 μl ketoset diluted 1:10 and inoculated IN with 50 μl undiluted virus, 6 mice/group. Mice were boosted with 50 μl virus diluted 1:2 in 199 media. Approximately one week later, mice were bled for serum. Neutralization titers were done in VERO cells.

All of the lines tested for neutralization antibodies had titers of at least 1:20. Line 19H had a titer of 1:80, however, WRSV had a titer of 1:320. The following tables further set forth the results of the immunogenicity study.

Table 5

Neutralization Titers (60% Reduction) without Complement†

Antiserum to Virus	Titers
19H	1:80
CRIa	1:40
Ca19V	1:20
CaBCV	1:40
Ca48V	1:40
WRSV	>1:320
Normal Serum	<1:20

†Serum periodate and heat treated (56° for 1 hour); serum diluted in presence of 2.5% FBS.

Table 6

ELISA Titers*

Antiserum to Virus	Titers+
19H	1:1280
CRIa	1:320
Ca19V	1:1280
CaBCV	1:2560
Ca48V	neg
WRSV	1:640

*Antigen - Whole RSV Virus
+Titers corrected for normal serum

C. **Immunogenicity Of RSV Lines (Study 3)**

A third study was performed to determine the immunogenicity of the following RSV lines: Ca19S, 19H, Ca19V, Ca48V, WRSV and Line 19 MRC5 28 25° (not deposited). Mice were infected with wild type and the RSV lines with twice boosted procedures, similar to the procedure described above. As shown in the table below, serum neutralization titers, carried out in the presence of complement, were reported as the reciprocal of the last dilution which reduced the number of viral plaques by 60%. Also shown in the table below, ELISA titers were reported as the reciprocal of the last dilution which had an OD reading of 0.1 or greater after correction for normal serum. All the lines tested for neutralization antibodies had titers of at least 320 and ELISA titers ranging from 160 to 40,960 after the second boost. Although line Ca19S produced the highest neutralization titer, all lines were similar in neutralizing ability and reached peak titer after the first boost. Line Ca19V produced the highest ELISA IgG titer. All RSV attenuated lines were immunogenic.

Table 7

Neutralization and ELISA Titers of Mice Immunized with Vaccine Lines

Viruses[a]	Titers Of Inoculum	Neutralization[b]			ELISA IgG[c]		
		2 Weeks[d]	4 Weeks	6 Weeks	2 Weeks	4 Weeks	6 Weeks
WRSV	5.3	80	640	320	160	2560	80
Line 19 MRC5 28 25°	5.7	80	640	1280	160	640	1280
Ca19S	7.5	80	640	640	320	2560	1280
19H	7.3	80	320	320	80	2560	320
Ca19V	6.3	320	640	640	320	5120	40960
Ca48V	7.4	80	320	320	160	5120	10240

[a]Viruses used were not plaque purified.
[b]Reported as reciprocal of last dilution which reduced viral plaques by 60%.
[c]Reported as reciprocal of last dilution which had an OD of 0.1 or greater after adjustment for normal serum.
[d]Weeks after Initial Vaccination.

D. Immunogenicity Of RSV Lines (Study 4)

A fourth study was performed to determine the immunogenicity of the following plaque purified RSV lines: 19HL 3PI, 19H 3PI, 19H MD and CRla MD. Pathogen-free BALB/c mice (approximately 8 weeks old) were immunized intranasally with either 2 X 10^4 $TCID_{50}$ of the lines or their respective progenitor viruses (designated wt 19 and WRSV), 2.0 X 10^4 pfu of mouse-adapted virus (designated live virus), or medium +5% FBS +5% glycerol (designated placebo). Animals were bled 4 weeks after the primary inoculation and boosted at 4 weeks with an equivalent dose of the vaccine formulation. Serum samples were also taken 4 weeks after the booster dose. All plaque purified lines elicited anti-F IgG antibodies at 4 and 8 weeks (see Figures 2A and 2B, wherein each value represents the mean titer of antisera from 6 animals). At the 8 week time point, the sera of animals that had received 2 doses of the various plaque purified lines had anti-RSV F IgG antibody titres that were comparable to that observed in the sera of mice that were immunized with the mouse-adapted virus (designated live virus). As shown in Figure 3 (values represent the mean titer of antisera from 6 animals), the sera of animals that were immunized with two doses of the various plaque purified lines had high levels of RSV-specific neutralizing antibodies. Thus, all RSV attenuated lines tested were immunogenic in the mouse model.

E. Boosting By Alternative Route of Administration

A study was performed to determine the effect of boosting by a route of administration that differs from the initial inoculation route of administration. Taconic Balb/c/AnNTacfBR 3 week old male mice were anesthetized IP with 200 μl ketoset diluted 1:10 in PBS and inoculated IN with 50 μl undiluted virus, WRSV (VERO 35°, Titer 2.00 x 10^6 Pfu's/ml in VERO cells), CRla, CaBCV, 19H and Ca48V. Mice were boosted with 100 μl virus via footpad injection. Approximately three weeks later, mice were boosted with 200 μl half virus and half complete Freund's adjuvant intramuscularly. Approximately ten days later, mice were bled for serum. Neutralization titers were done in VERO cells in the presence of complement. The following table sets forth the results of the study.

Table 8

Neutralization Data in Presence of Complement (60% Reduction)

Antiserum to:	Titer with Complement*
Normal Serum	
19H	1:160
CaBCV	1:160
Ca48V	>1:640
CRIa	>1:640
WRSV	>1:640

*Titer reported as last dilution with 60% reduction over positive controls with complement average positive control = 100.33 colonies/well.

F. Cytotoxicity Study

Generation of CTL. Spleens from two BALB/c mice from each group that were immunized with either live mouse adapted A2 virus, line 19H or placebo, (see A. above, Immunogenicity of RSV Lines (Study 1)), were removed three weeks after the booster dose. Single cell suspensions were prepared and incubated at 2.5×10^7 cells in RPMI 1640 plus 10% FBS. Gamma-irradiated (3,000 rads) syngeneic spleen cells were infected with RSV at an MOI of 1 for 2 h. The cells were washed twice to remove free virus and 2.5×10^7 spleen cells in a final volume of 10 ml of complete medium. CTL activity was tested 5-6 days following re-stimulation.

Cytoxicity assay. On the date of the assay, effector cells were washed twice with fresh medium and viable cell counts were determined by the Trypan blue dye exclusion method. BC cells (2×10^6 cells), a BALB/c fibroblast cell line, as well as BCH4 cells (2×10^6 cells), a BALB/c fibroblast T cell line persistently infected with RSV, were pulsed with 200 μCi of Sodium ^{51}chromate (Dupont) for 90 min. The targets were washed three times with medium to remove free ^{51}chromium. Viable cell counts of the target cells were determined and target cell suspensions were prepared at 2×10^4 cells/mL. Washed responder T-cells (in 100 μl) were incubated with 2×10^3 target cells (in 100 μl) at various Effector:Target cell ratios in triplicate in 96-well V-bottomed tissue-culture plates for 4 h at 37°C with 6% CO_2. Spontaneous and total release of ^{51}chromium were determined by incubating target cells with either medium or 2.5% Triton-X100 in the absence of responder lymphocytes. Six replicates of each were prepared. After 4 h plates were centrifuged at 200 x g for 2 min and 100 μl supernatant was removed from each well to determine the amount of ^{51}chromium released. Percentage specific ^{51}chromium release was calculated as (Experimental Release - Spontaneous Release) (Total Release - Spontaneous Release) x 100. The

Spontaneous Release of ^{51}chromium in the absence of effector cells was found to be between 10-15% in these studies.

Figure 4 shows the results of the study. In Figure 4, lysis of BC (filled symbols) and BCH4 (empty symbols) by CTL generated from BALB/c mice immunized with placebo (Triangle), live RSV (Square) or line 19H (Circle) is shown. Mice immunized with live RSV (empty square) or 19H (empty circle) lysed BCH4 cells (RSV infected) significantly at all effector to target cell ratios when compared to the lysis of BC (un-infected) cells. There was no significant levels of lysis by effector cells from the placebo indicating that line 19H is capable of inducing significant levels of CTL activity.

G. Protection Study

Generation of CTL. Spleens from two BALB/c mice from each group that were immunized with either the mutants or their respective progenitor viruses, live mouse adapted virus or medium (placebo) were removed three weeks after the booster dose. Single cell suspensions were prepared and incubated at 2.5 X 10^7 cells in RPMI 1640 plus 10% FBS. Gamma-irradiated (3,000 rads) syngeneic spleen cells were infected with RSV at an MOI of 1 for 2h. The cells were washed twice to remove free virus and 2.5 X 10^7 spleen cells in a final volume of 10 mL of complete medium. CTL activity was tested 5-6 days following re-stimulation.

Cytotoxicity assay. On the day of the assay, effector cells were washed twice with fresh medium and were resuspended in 2 mL of complete medium. BC cells (2 X 10^6 cells), a BALB/c fibroblast cell line, as well as BCH4 cells (2 X 10^6 cells), a BALB/c fibroblast T cell line persistently infected with RSV, were pulsed with 200μCi of Sodium ^{51}chromate (Dupont) for 90 min. The targets were washed three times with medium to remove free ^{51}chromium. Viable cell counts of the target cells were determined and target cell suspensions were pared at 2 X 10^4 cells/mL. Washed responder T-cells at various dilutions (in 100 μl) were incubated with 2 X 10^3 target cells (in 100 μl) in triplicate in 96-well V bottomed tissue-culture plates for 4 h at 37°C with 6% CO_2. Spontaneous and total release of ^{51}chromium were determined by incubating target cells with either medium of 2.5% Triton -X100 in the absence of responder lymphocytes. Six replicates of each were prepared. After 4 h plates were centrifuged at 200 X g for 2 min. and 100μl of supernatant was removed from each well to determine the amount of ^{51}chromium related. Percentage specific ^{51}chromium release was calculated as (Experimental Release-Spontaneous Release)/ (Total release - Spontaneous release) X 100. The spontaneous release of ^{51}chromium in

the absence of effector cells was found to be between 10-15% in these studies. The lysis in cultures is directly proportional to the number of effector cells present in the culture, which in turn is proportional to the number of CTL precursors activated *in vivo* by that particular immunogen.

The results of this cytotoxicity study are shown in Figure 5. In Figure 5, lysis of BC (open symbols) and BCH4 cells (filled symbols) by CTL generated from BALB/c mice immunized with either placebo, 19HL 3PI, 19H 3PI, 19H MD, wt 19, CRla MD or WRSV, or live mouse adapted virus is shown. Mice immunized with live mouse adapted RSV, 19HL 3PI, 19H 3PI, 19H MD, CRla MD, wt 19 and WRSV, lysed BCH4 cells (RSV infected) at all effector cell dilutions when compared to the lysis of BC (non-infected) cells. There were no significant level of lysis by effector cells from the placebo indicating that all the tested viruses are capable of inducing significant levels of CTL activity.

To evaluate the ability of the plaque purified deposited viruses to protect mice against live virus challenge, mice that were immunized with either the plaque purified viruses, progenitor viruses or medium alone (see D. above, Immunogenicity of RSV Lines (Study 4)), were challenged with 10^6 pfu of RSV A2 immediately after the 8 week bleed. Lungs were harvested four days after virus challenge and virus titers in lung homogenates were determined by the plaque assay. As shown in the table below, mice immunized with the viruses of the present invention were protected against live virus challenge. The protective ability was comparable to that observed with mice that were inoculated with live mouse adapted virus.

Table 9

Protective Ability Of The Plaque Purified Viruses And Progenitor Viruses

Virus	Mean Virus Lung Titre (log pfu/g ± s.d.)*	% Animals Protected
Placebo	5.2 ± .06	0
19HL 3PI	≤ 1.7 ± 0	100
19H 3PI	≤ 1.7 ± 0	100
19H MD	≤ 1.7 ± 0	100
wt 19	≤ 1.7 ± 0	100
CRIa MD	≤ 1.7 ± 0	100
WRSV	1.9 ± 2.2	50
Live virus	≤ 1.7 ± 0	100

*Represents the mean value of 6 animals.

SPECIFIC EXAMPLE 4 - SEQUENCE ANALYSIS OF THE F GENE

A. Sequence Comparison - WRSV

In identifying the molecular basis for the *ts* phenotype, the F gene of the wild type (WRSV), line Ca19V and line 19H were sequenced using polymerase chain reaction (PCR). The F gene is composed of 1899 nucleotides, 13 of which are non-coding at the 3′ end. Both viruses were grown in Vero cells to isolate the RNA for sequencing. Comparison of the F genes of line Ca19V and line 19H revealed 73 nucleotide and 15 amino acid differences. Comparison of the F genes of line 19H and WRSV revealed 72 nucleotide and 13 amino acid differences. There are 11 nucleotide changes and 6 amino acid changes between the F genes of the two line 19 attenuated viruses, Ca19V and 19H. Only base changes (no insertions or deletions) were found. The F genes of the two attenuated line 19 viruses have 66 nucleotides and 11 amino acids in common but differ from that of WRSV (amino acid positions 66, 76, 79, 97, 119, 129, 191, 357, 384, 522 and 530).

The Garnier Osguthorpe Robson (GOR) predicted F protein structures of the two line 19 viruses are nearly identical; however, the GOR F protein structure of WRSV differs at amino acid 97, 119, 191, 357 and 522 from both attenuated viruses and differs at amino acid 294 only from line Ca19V. Amino acid 97 (threonine in both line 19's and methionine in WRSV) predicts a turn in the attenuated line 19 viruses

not present in WRSV. Amino acid 119 (phenylalanine in both line 19's and leucine in WRSV) predicts an additional turn in the attenuated line 19 viruses not present in WRSV. Amino acid 191 (lysine in the attenuated lines 19 viruses and arginine in WRSV) predicts the formation of an alpha helix in the attenuated line 19 viruses while WRSV continues a beta sheet fold and then turns. Amino acid 357 predicts the formation of an alpha helix in WRSV not present in either attenuated line 19 virus and amino acid 522 predicts a turn in WRSV not present in either attenuated line 19 viruses. Amino acid 294 predicts the formation of a beta sheet in WRSV and 19H not present in Ca19V. It is interesting that, although there are 6 amino acid differences between the two attenuated line 19 viruses, the 2 attenuated viruses have the same predicted protein fold whereas the WRSV fold is quite different. Thus, amino acids 97, 119, 191, 357 and 522 are good candidates for attenuating lesions in the F protein.

Table 10

Sequence Comparison of the Genes Coding for the F Proteins of WRSV, Ca19V and 19H

Nucleo-tide #	WRSV	Ca19V	19H	Amino Acid #	WRSV	Ca19V	19H
76	C	T	T				
85	T	C	C				
106	A	G	G				
131	***G***	***G***	***A***	***40***	***Val***	***Val***	***Ile***
155	C	T	T				
199	T	C	C				
209	A	G	G	66	Lys	Glu	Glu
220	T	C	C				
235	T	C	C				
240	T	C	C	76	Val	Ala	Ala
250	G	A	A	79	Met	Ile	Ile
265	T	C	C				
271	T	C	C				
296	T	C	C				
303	T	C	C	<u>97</u>	<u>Met</u>	<u>Thr</u>	<u>Thr</u>
322	A	T	T				
352	***G***	***G***	***A***				

Nucleo-tide #	WRSV	Ca19V	19H	Amino Acid #	WRSV	Ca19V	19H
368	C	T	T	119	Leu	Phe	Phe
370	C	T	T				
398	T	A	A	129	Leu	Ile	Ile
430	T	C	C				
457	C	T	T				
505	G	A	A				
511	G	A	A				
514	C	A	A				
541	G	A	A				
585	G	A	A	191	Arg	Lys	Lys
604	C	T	T				
622	G	A	A				
623	T	C	C				
716	***A***	***G***	***C***	***235***	***Arg***	***Gly***	***Arg***
718	G	A	A				
763	C	T	T				
787	G	A	A				
871	C	T	T				
893	***G***	***A***	***G***	***294***	***Glu***	***Lys***	***Glu***
898	A	G	G				
906	***C***	***C***	***A***	***298***	**Ala**	***Ala***	***Glu***
959	T	C	C				
961	A	G	G				
1003	A	C	C				
1015	A	G	G				
1048	T	C	C				
1057	A	T	T				
1083	A	C	C	357	Lys	Thr	Thr
1087	T	C	C				
1090	A	G	G				
1116	***A***	***T***	***A***	***368***	***Asp***	***Val***	***Asp***

Nucleo-tide #	WRSV	Ca19V	19H	Amino Acid #	WRSV	Ca19V	19H
1126	C	T	T				
1163	G	A	A	384	Val	Ile	Ile
1180	***C***	***C***	***T***				
1206	***C***	***T***	***C***	***398***	***Ser***	***Leu***	***Ser***
1222	***C***	***T***	***C***				
1228	C	T	T				
1241	*C*	*C*	*T*				
1246	A	G	G				
1465	C	A	A				
1501	T	C	C				
1504	G	A	A				
1519	T	C	C				
1520	T	C	C				
1576	A	C	C				
1577	A	G	G	<u>522</u>	<u>Thr</u>	<u>Ala</u>	<u>Ala</u>
1603	A	G	G	530	Ile	Met	Met
1655	***C***	***T***	***C***				
1669	C	A	A				
1705	G	A	A				
1739	A	T	T				
1751	C	T	T				
1781	C	A	A				
1802	G	A	A				
1813	T	C	C				
1829	T	C	C				
1838	A	G	G				
1841	C	T	T				
1843	T	C	C				
1847	T	C	C				

Differences between Ca19V and 19H are italicized and bolded. Amino acids which impact on the predicted protein folds are underlined.

B. Sequence Comparison - wt 19

The nucleotide sequence of the F genes of line 19 wild type (wt 19), Ca19V and 19H, were compared by *f-mol* sequencing. The following table lists the nucleotide and amino acid changes in the F genes between lines Ca19V, 19H and wt 19.

Table 11

Sequence Differences Between The F Genes Of wt 19, Ca19V and 19H

Nucleo-tide #	wt 19	19H	Ca19V	Amino Acid #	wt 19	19H	Ca19V
131	G	**A**	G	40	val	**iso**	val
352	A	A	*G*				
716	C	C	*G*	235	arg	arg	*gly*
893	G	G	*A*	294	glu	glu	*lys*
906	C	**A**	C	298	ala	**glu**	ala
1116	A	A	*T*	368	asp	asp	*val*
1180	T	T	*C*				
1206	C	C	*T*	398	ser	ser	*leu*
1222	T	**C**	T				
1241	T	T	*C*				
1249	C	**A**	*A*				
1655	C	C	*T*				

Differences between 19H and wt 19 are bolded.
Differences between Ca19V and wt 19 are italicized.

Between wt 19 and 19H, there were 4 nucleotide differences, 2 of which coded for amino acid differences. Amino acid 40, valine in wt 19 and isoleucine in 19H, is a conserved change since both are hydrophobic and neither is charged. The difference at amino acid 298 is not conserved. Alanine, in wt 19, is hydrophobic and not charged while glutamic acid in 19H, is not hydrophobic and is negatively charged. Chou Fasman analysis predicts that the glutamic acid of 19H extends an alpha helix thus postponing the formation of a beta sheet predicted by the alanine at 298 of the wt 19 F protein.

Between wt 19 and Ca19V, there are 9 nucleotide differences and 4 amino acid differences, none of which is a conserved change. Amino acid 235 is basic arginine to uncharged glycine; amino acid 294 is negatively charged glutamic acid to positively charged lysine; amino acid 368 is negatively charged aspartic acid to hydrophobic valine; and amino acid 398 is uncharged serine to hydrophobic leucine. The structure of the two F proteins, as predicted by Chou Fasman, differs only at amino acid 234; Ca19V extends an alpha helix, thus losing a turn predicted for the wt 19 F protein.

None of the amino acid differences is shared by the two line 19 attenuated viruses, but nucleotide 1249 (which does not code for an amino acid change) is C in wt 19 and A in both Ca19V and 19H.

SPECIFIC EXAMPLE 5 - PLAQUE PURIFICATION

As indicated above, several of the deposited strains were plaque purified. In particular, Line 19HL 3PI was passed 72 times in MRC5 cells, then plaque purified three times in Vero cells. Line 19H 3PI was passed 70 times in MRC5 cells, then plaque purified three times in Vero cells and passed 2 times in MRC5 cells. Line 19H MD was passed 92 times in MRC5 cells, then purified by limiting dilution three times. Line CRIa MD was passed 28 times at 25°C and 1 time at 33°C then purified by limiting dilution five times. The following table sets forth the titers of the strains at 33°C and 39°C, illustrating temperature sensitivity.

Table 12

$TCID_{50}$ In MRC5 Cells

Virus	33°C	39°C
19HL 3PI	1.0×10^6	1.0×10^5
19H 3PI	3.2×10^5	2.0×10^3
19H MD	3.2×10^5	1.0×10^3
CRIa MD	$>1.0 \times 10^6$	2.0×10^3
19H	3.16×10^6	
19H 4MD	3.16×10^4	
WRSV	2.0×10^5	2.0×10^5

SPECIFIC EXAMPLE 6 - HUMAN STUDIES

The attenuated virus of the present invention is administered to human subjects according to well established human RSV protocols, for example, those described in Wright et al., *Infect. Immun.* 37:397-400 (1982); Kim et al., *Pediatrics* 52:56-63 (1973) and Wright et al., *J. Pediatr.* 88:931-936 (1976). Briefly, adults or children are inoculated intranasally via droplet with 10^2 to 10^9 PFU, preferably 10^4 to 10^5 PFU, of attenuated virus per ml in a volume of 0.5 ml. Antibody response is evaluated by complement fixation, plaque neutralization, and/or enzyme-linked immunosorbent assay. Individuals are monitored for signs and symptoms of upper respiratory illness. Subsequent immunizations are administered periodically to the individuals as necessary to maintain sufficient levels of protective immunity.

The foregoing discussion discloses and describes merely exemplary embodiments of the present invention. One skilled in the art will readily recognize from such discussion, and from the accompanying claims, that various changes, modifications and variations can be made therein without departing from the spirit and scope of the invention as defined in the following claims.

All patents and other references cited herein are expressly incorporated by reference.

WE CLAIM:

1. A respiratory syncytial virus selected from the group consisting of the viruses having ATCC Designation Nos. VR-2511, VR-2512, VR-2513, VR-2514, VR-2515, VR-2516, VR-2517, VR-2564, VR-2565, VR-2566, VR-2567 and VR-2572, and derivative viruses thereof.

2. The attenuated virus of Claim 1 for use as an active pharmaceutical substance.

3. The use of the attenuated virus of Claim 1 for the preparation of a medicament for the treatment or prevention of disease caused by infection by respiratory syncytial virus.

4. A method of producing a vaccine against disease caused by infection by respiratory syncytial virus, comprising:

a) administering the virus of Claim 1 to a test host to determine an amount and a frequency of administration thereof to elicit a protective immune response in said host; and

b) formulating said virus in a form suitable for administration to a treatable host in accordance with said determined amount and frequency of administration.

5. A method of determining the presence of antibodies specifically reactive with a respiratory syncytial virus in a sample comprising the steps of:

a) contacting the sample with the virus of Claim 1 to produce complexes comprising the virus and antibodies present in the sample specifically reactive therewith; and

b) determining production of the complexes.

6. A method of determining the presence of a respiratory syncytial virus in a sample comprising the steps of:

   a) contacting the sample with an antibody specifically reactive with a respiratory syncytial virus of Claim 1 to produce complexes comprising the antibody and the virus present in the sample specifically reactive therewith; and

   b) determining production of the complexes.

7. A nucleic acid molecule encoding the respiratory syncytial virus of Claim 1 and equivalent nucleic acid molecules thereof.

8. A vaccine composition comprising an attenuated respiratory syncytial virus and a pharmaceutically acceptable carrier.

9. The vaccine composition of Claim 8, wherein the attenuated respiratory syncytial virus is selected from the group consisting of the viruses of Claim 1 and derivative viruses thereof.

10. A method of immunizing a host against disease caused by infection by respiratory syncytial virus which comprises administering to the host an immunoeffective amount of the vaccine of Claim 8 or the nucleic acid molecule of Claim 7.

11. An attenuated respiratory syncytial virus characterized by having a codon encoding an amino acid in the F protein chosen from the group consisting of a codon encoding isoleucine at amino acid 40, a codon encoding glutamic acid at amino acid 66, a codon encoding alanine at amino acid 76, a codon encoding isoleucine at amino acid 79, a codon encoding threonine at amino acid 97, a codon encoding phenylalanine at amino acid 119, a codon encoding isoleucine at amino acid 129, a codon encoding lysine at amino acid 191, a codon encoding glycine at amino acid 235, a codon encoding arginine at amino acid 235, a codon encoding lysine at amino acid 294, a codon encoding glutamic acid at amino acid 294, a codon encoding glutamic acid at amino acid 298, a codon encoding alanine at amino acid 298, a codon encoding threonine at amino acid 357, a codon encoding valine at amino acid 368, a codon encoding aspartic acid at amino acid 368, a codon encoding isoleucine at amino acid 384, a codon encoding leucine at amino acid 398, a codon encoding serine at amino acid 398, a codon encoding alanine at amino acid 522 and a codon encoding methionine at amino acid 530.

12. An attenuated respiratory syncytial virus characterized by having a codon encoding an amino acid in the F protein chosen from the group consisting of a codon encoding isoleucine at amino acid 40, a codon encoding glycine at amino acid 235, a codon encoding arginine at amino acid 235, a codon encoding lysine at amino acid 294, a codon encoding glutamic acid at amino acid 294, a codon encoding glutamic acid at amino acid 298, a codon encoding alanine at amino acid 298, a codon encoding valine at amino acid 368, a codon encoding aspartic acid at amino acid 368, a codon encoding leucine at amino acid 398 and a codon encoding serine at amino acid 398.

13. A respiratory syncytial virus selected from the group consisting of wt 19, and WRSV, and derivative viruses thereof.

14. The virus of Claim 13, wherein the derivative virus is attenuated.

15. The attenuated virus of Claim 14 for use as an active pharmaceutical substance.

16. The use of the attenuated virus of Claim 14 for the preparation of a medicament for the treatment or prevention of disease caused by infection by respiratory syncytial virus.

17. A method of producing a vaccine against disease caused by infection by respiratory syncytial virus, comprising:

a) administering the virus of Claim 14 to a test host to determine an amount and a frequency of administration thereof to elicit a protective immune response in said host; and

b) formulating said virus in a form suitable for administration to a treatable host in accordance with said determined amount and frequency of administration.

18. A method of determining the presence of antibodies specifically reactive with a respiratory syncytial virus in a sample comprising the steps of:

a) contacting the sample with the virus of Claim 14 to produce complexes comprising the virus and antibodies present in the sample specifically reactive therewith; and

b) determining production of the complexes.

19. A method of determining the presence of a respiratory syncytial virus in a sample comprising the steps of:

a) contacting the sample with an antibody specifically reactive with a respiratory syncytial virus of Claim 14 to produce complexes comprising the antibody and the virus present in the sample specifically reactive therewith; and

b) determining production of the complexes.

20. A nucleic acid molecule encoding the respiratory syncytial virus of Claim 13 and equivalent nucleic acid molecules thereof.

21. The vaccine composition of Claim 8, wherein the attenuated respiratory syncytial virus is selected from the group consisting of the viruses of Claim 14 and derivative viruses thereof.

22. A method of immunizing a host against disease caused by infection by respiratory syncytial virus which comprises administering to the host an immunoeffective amount of the vaccine of Claim 21 or the nucleic acid molecule of Claim 20.

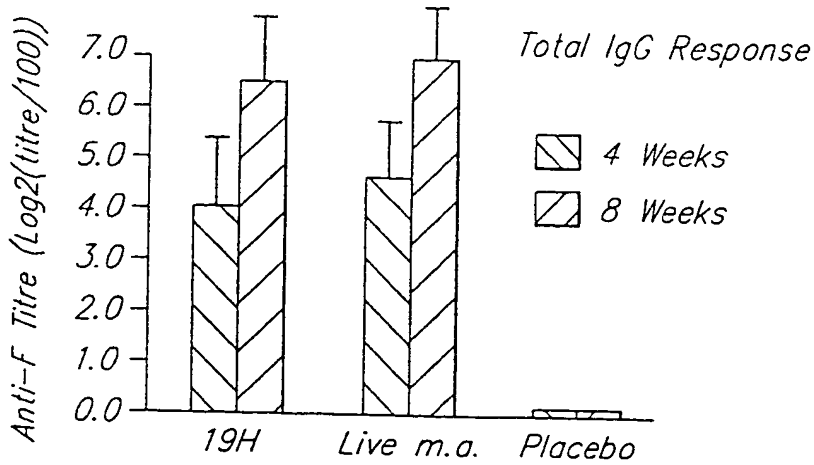

FIG. 1A.

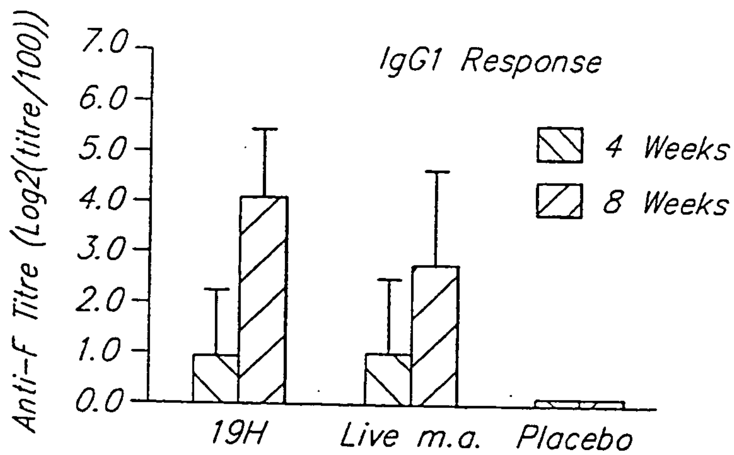

FIG. 1B.

FIG. 1C.

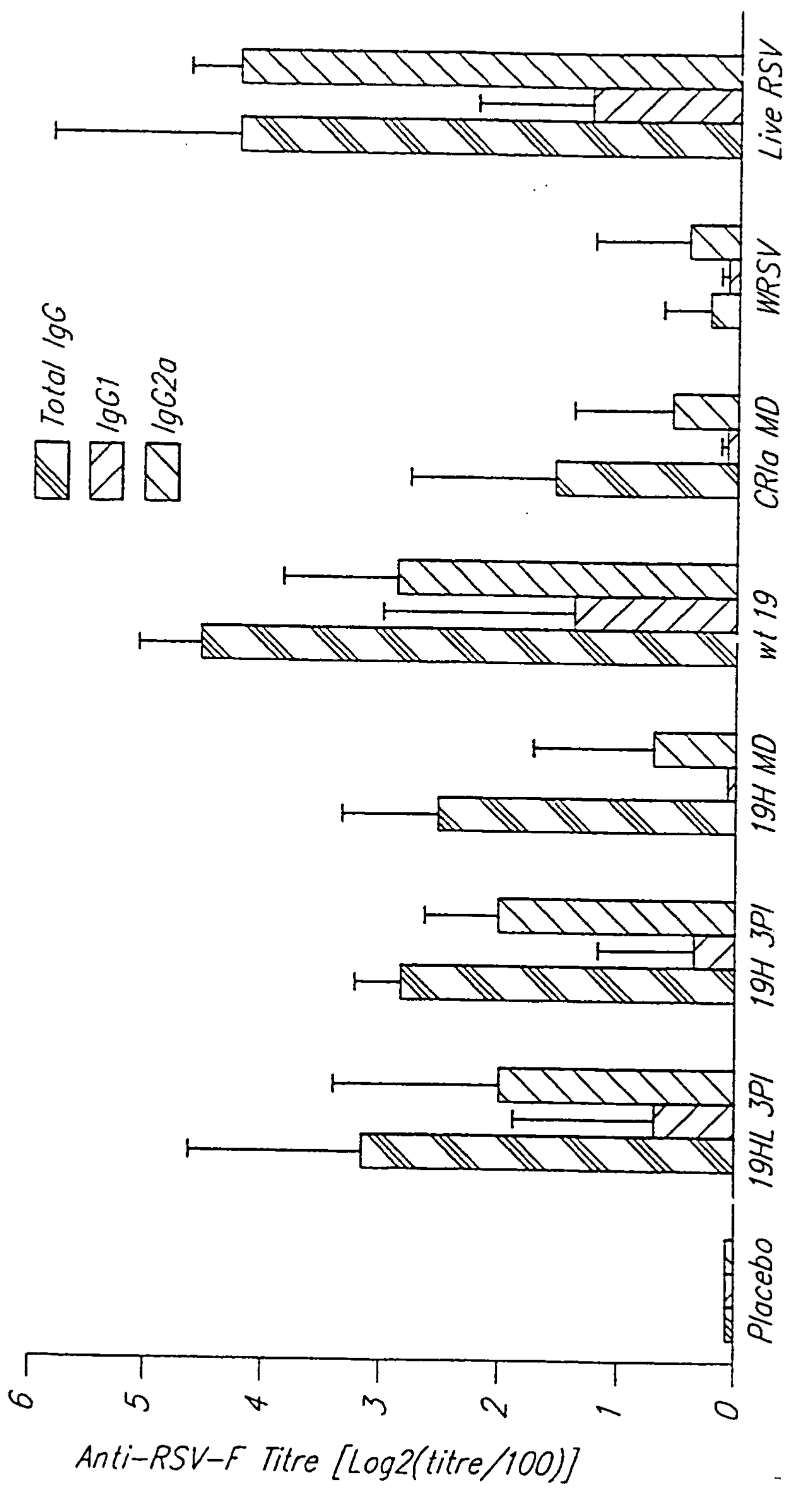
Total IgG
IgG1
IgG2a
Placebo
19HL 3PI
19H 3PI
19H MD
wt 19
CRIa MD
WRSV
Live RSV
0
1
2
3
4
5
6
Anti-RSV-F Titre [Log2(titre/100)]

FIG. 2A.

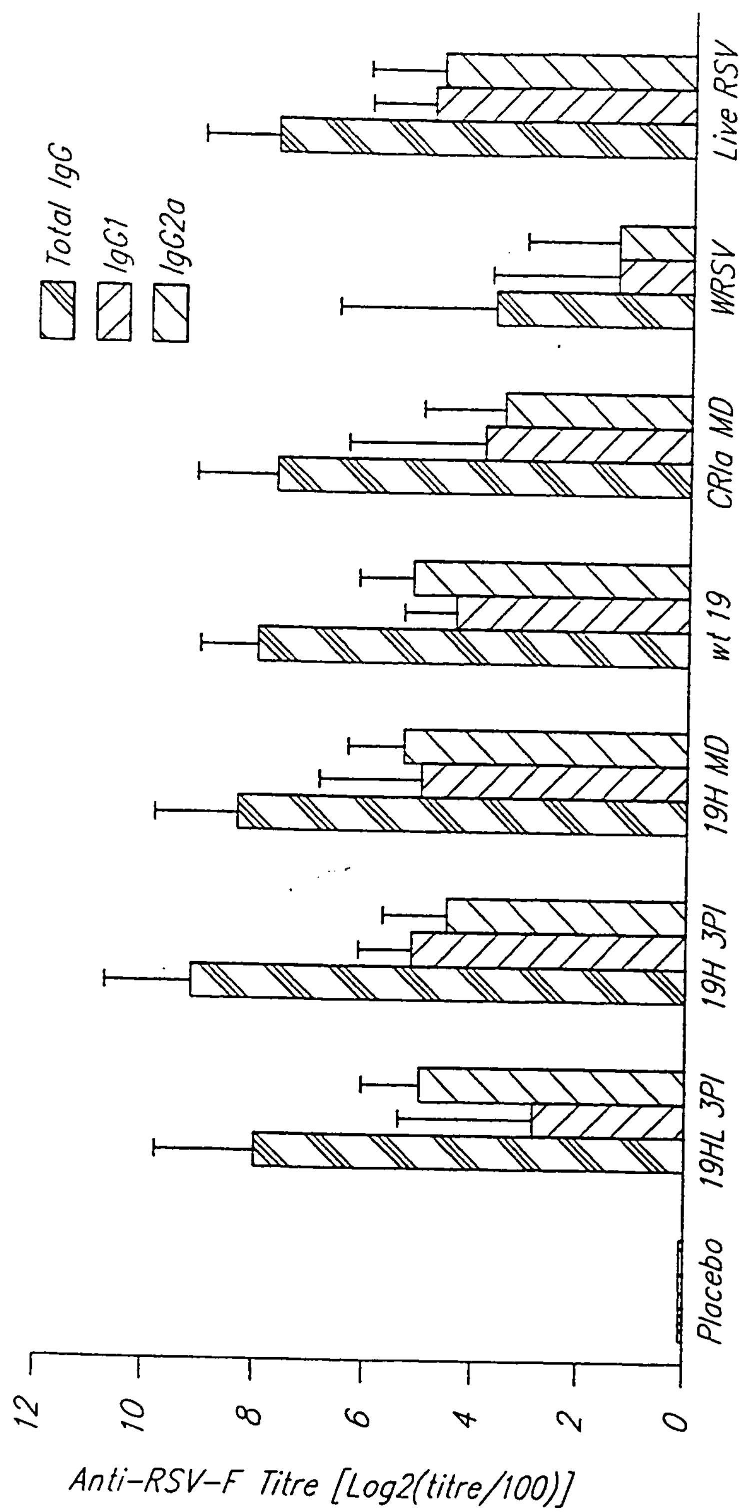
Total IgG
IgG1
IgG2a
Placebo
19HL 3PI
19H 3PI
19H MD
wt 19
CRIa MD
WRSV
Live RSV
0
2
4
6
8
10
12
Anti-RSV-F Titre [Log2(titre/100)]

FIG. 2B.

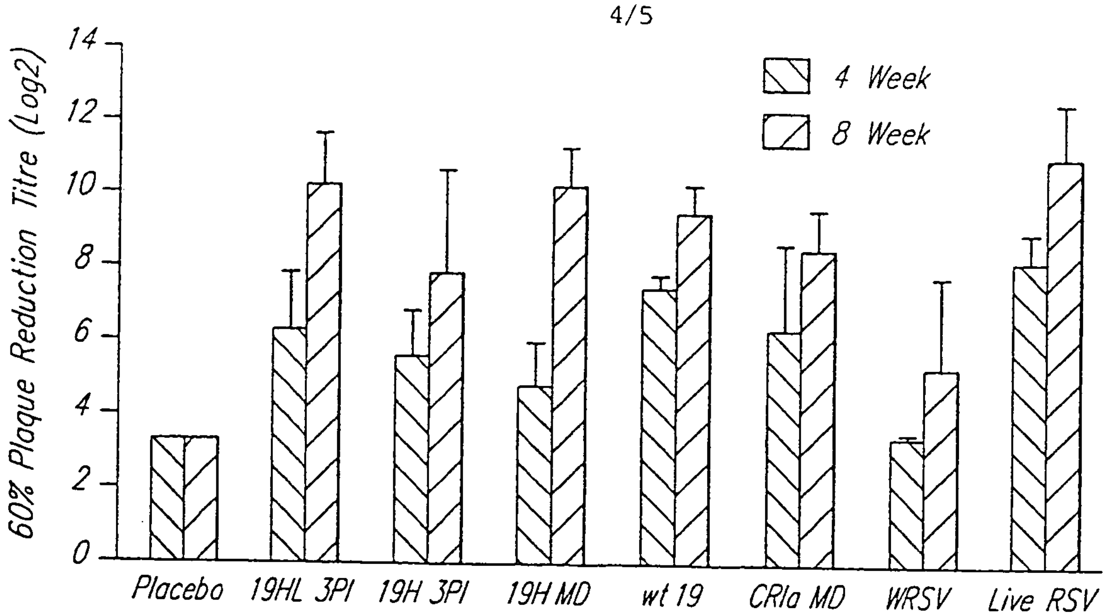
14
12
10
8
6
4
2
0
60% Plaque Reduction Titre (Log2)
4 Week
8 Week
Placebo
19HL 3PI
19H 3PI
19H MD
wt 19
CRIa MD
WRSV
Live RSV

FIG. 3.

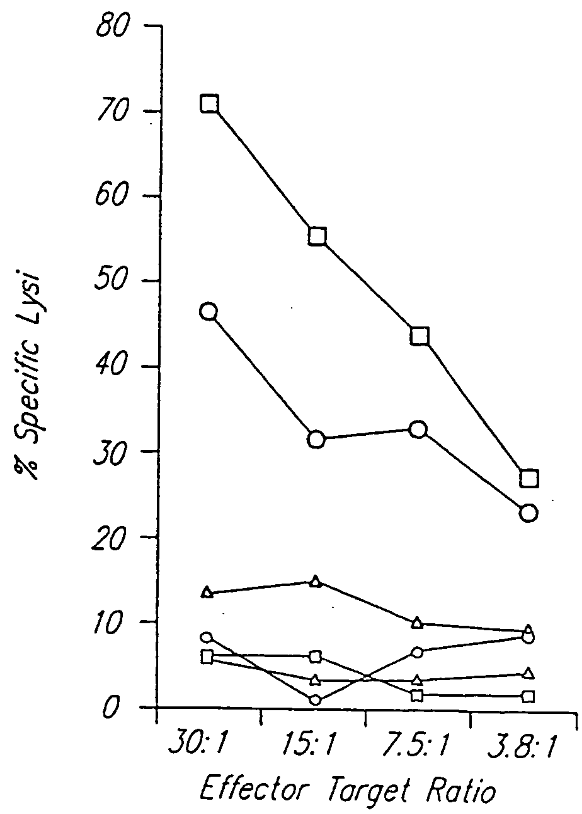
80
70
60
50
40
30
20
10
0
% Specific Lysi
30:1
15:1
7.5:1
3.8:1
Effector Target Ratio

FIG. 4.

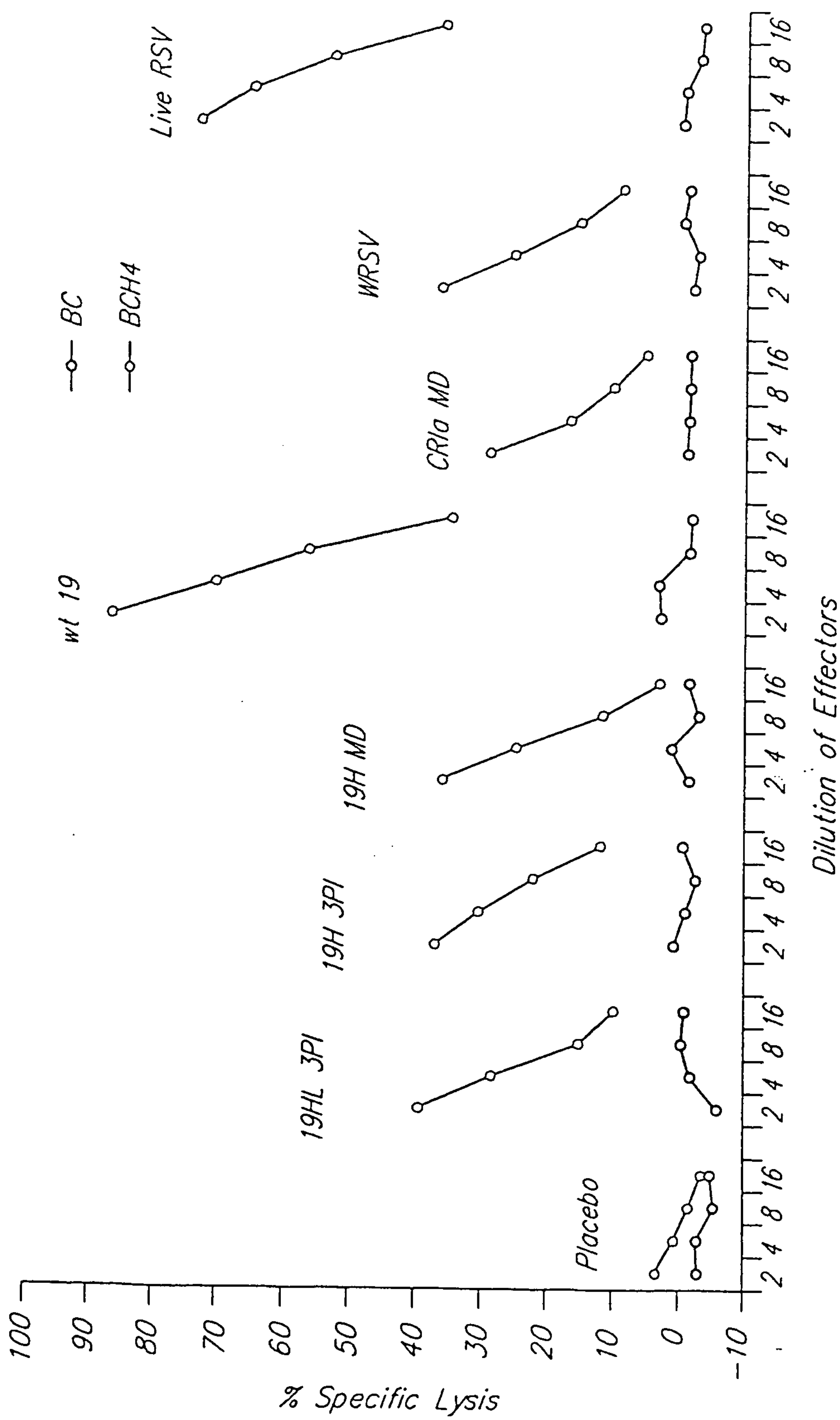
BC
BCH4
Live RSV
WRSV
CRIa MD
wt 19
19H MD
19H 3PI
19HL 3PI
Placebo
100 90 80 70 60 50 40 30 20 10 0 −10
% Specific Lysis
2 4 8 16
Dilution of Effectors

FIG. 5

Applicant's or agent's file reference number	2115S01113POC	International application No. PCT/US 98/06636

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on pages 4 and 5, line

B. IDENTIFICATION OF DEPOSIT RSV, 1a-CRSV-5 CL 15 MRC27 — Further deposits are identified on an additional sheet [X]

Name of depositary institution

American Type Culture Collection

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
20 September 1995 (20.09.95)	VR-2511 (see additional sheet)

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet [ ]

In respect of the designation of Finland, samples of the deposited micro-organisms will be made available until the application has been laid open to public inspection (by the National Board of Patents and Registration), or has been finally decided upon by the National Board of Patents and Registration without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

Finland

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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[ ] This sheet was received with the international application	[ ] This sheet was received by the International Bureau on:
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Continuation Of Indications Relating To A Deposited Microorganism
(PCT Rule 13*bis*)
For EPO

Identification Of Deposit	American Type Culture Collection Accession Number	Date of Deposit
RSV, Line 19 MRC5-15-25° st-33°	VR-2512	20 September 1995 (20.09.95)
RSV, Line 19 MRC5-60-35°	VR-2513	20 September 1995 (20.09.95)
RSV, Line 48 MRC5-14-25° st MRC1-33° VERO 10-25° VERO 1-33°	VR-2514	20 September 1995 (20.09.95)
RSV, Line 19 MRC5-10-25° VERO16-25° VERO 6-20° VERO 3-33°	VR-2515	20 September 1995 (20.09.95)
RSV, CRSV-BC5 CL-17 MRC30	VR-2516	20 September 1995 (20.09.95)
RSV, CRSV-BC13 MRC19-25° MRC1-33°	VR-2517	20 September 1995 (20.09.95)
RSV, Line 19 MRC5 92-35°C, Clone 4-1, MRC5, P-103-33° Purified by minimal limited dilution (MLD)	VR-2567	2 April 1997 (02.04.97)
RSV, Line 19 MRC 70-35°C, Vero 3-35°C, MRC5 2-35°C Clone 2-35°C (3PI) Plaque purified	VR-2564	2 April 1997 (02.04.97)
RSV, Line 19 MRC5, 92-35°C Clone 5-1 Purified by MLD	VR-2565	2 April 1997 (02.04.97)
RSV, la-CRSV-5 MRC-38-25°C, MRC1-33°C Purified by MLD	VR-2566	2 April 1997 (02.04.97)
RSV, Line 19 MRC5 72-35°C VERO 3-35°C Large Clone 6-35°C, (3PI) Plaque Purified	VR-2572	23 April 1997 (23.04.97)
Progenitor wt RSV, line 19 MRC5 1-33°C, VERO 2-35°C, MRC5 1-35°C Purified by MLD	VR-2570	23 April 1997 (23.04.97)
Progenitor wt RSV, WI 38-3 MRC5 9-35°C, MRC5 1-35°C, Purified by MLD	VR-2571	23 April 1997 (23.04.97)

Applicant's or agent's file reference number	2115S01113POC	International application No. PCT/US 98/06536

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on pages 4 and 5 , line

B. IDENTIFICATION OF DEPOSIT RSV, 1a-CRSV-5 CL 15 MRC27 Further deposits are identified on an additional sheet [X]

Name of depositary institution

American Type Culture Collection

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
20 September 1995 (20.09.95)	VR-2511 (see additional sheet)

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

In respect of the designation of Singapore, the furnishing of a sample of a microorganism shall only be made available to an expert.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

Singapore

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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Continuation Of Indications Relating To A Deposit d Microorganism
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For EPO

Identification Of Deposit	American Type Culture Collection Accession Number	Date of Deposit
RSV, Line 19 MRC5-15-25° st-33°	VR-2512	20 September 1995 (20.09.95)
RSV, Line 19 MRC5-60-35°	VR-2513	20 September 1995 (20.09.95)
RSV, Line 48 MRC5-14-25° st MRC1-33° VERO 10-25° VERO 1-33°	VR-2514	20 September 1995 (20.09.95)
RSV, Line 19 MRC5-10-25° VERO16-25° VERO 6-20° VERO 3-33°	VR-2515	20 September 1995 (20.09.95)
RSV, CRSV-BC5 CL-17 MRC30	VR-2516	20 September 1995 (20.09.95)
RSV, CRSV-BC13 MRC19-25° MRC1-33°	VR-2517	20 September 1995 (20.09.95)
RSV, Line 19 MRC5 92-35°C, Clone 4-1, MRC5, P-103-33° Purified by minimal limited dilution (MLD)	VR-2567	2 April 1997 (02.04.97)
RSV, Line 19 MRC 70-35°C, Vero 3-35°C, MRC5 2-35°C Clone 2-35°C (3PI) Plaque purified	VR-2564	2 April 1997 (02.04.97)
RSV, Line 19 MRC5, 92-35°C Clone 5-1 Purified by MLD	VR-2565	2 April 1997 (02.04.97)
RSV, Ia-CRSV-5 MRC-38-25°C, MRC1-33°C Purified by MLD	VR-2566	2 April 1997 (02.04.97)
RSV, Line 19 MRC5 72-35°C VERO 3-35°C Large Clone 6-35°C, (3PI) Plaque Purified	VR-2572	23 April 1997 (23.04.97)
Progenitor wt RSV, line 19 MRC5 1-33°C, VERO 2-35°C, MRC5 1-35°C Purified by MLD	VR-2570	23 April 1997 (23.04.97)
Progenitor wt RSV, WI 38-3 MRC5 9-35°C, MRC5 1-35°C, Purified by MLD	VR-2571	23 April 1997 (23.04.97)

Applicant's or agent's file reference number	⌐115S01113PCC	International application No. PCT/US 98/06636

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on pages 4 and 5, line

B. IDENTIFICATION OF DEPOSIT RSV, 1a-CRSV-5 CL 15 MRC27 — Further deposits are identified on an additional sheet [X]

Name of depositary institution

American Type Culture Collection

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
20 September 1995 (20.09.95)	VR-2511 (see additional sheet)

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet [ ]

In respect of the designation of Sweden, samples of the deposited microorganisms will be made available until the application has been laid open to public inspection (by the Swedish Patent Office), or has been finally decided upon by the Swedish Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

Sweden

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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For Sweden

Identification Of Deposit	American Type Culture Collection Accession Number	Date of Deposit
RSV, Line 19 MRC5-15-25° st-33°	VR-2512	20 September 1995 (20.09.95)
RSV, Line 19 MRC5-60-35°	VR-2513	20 September 1995 (20.09.95)
RSV, Line 48 MRC5-14-25° st MRC1-33° VERO 10-25° VERO 1-33°	VR-2514	20 September 1995 (20.09.95)
RSV, Line 19 MRC5-10-25° VERO16-25° VERO 6-20° VERO 3-33°	VR-2515	20 September 1995 (20.09.95)
RSV, CRSV-BC5 CL-17 MRC30	VR-2516	20 September 1995 (20.09.95)
RSV, CRSV-BC13 MRC19-25° MRC1-33°	VR-2517	20 September 1995 (20.09.95)
RSV, Line 19 MRC5 92-35°C, Clone 4-1, MRC5, P-103-33° Purified by minimal limited dilution (MLD)	VR-2567	2 April 1997 (02.04.97)
RSV, Line 19 MRC 70-35°C, Vero 3-35°C, MRC5 2-35°C Clone 2-35°C (3PI) Plaque purified	VR-2564	2 April 1997 (02.04.97)
RSV, Line 19 MRC5, 92-35°C Clone 5-1 Purified by MLD	VR-2565	2 April 1997 (02.04.97)
RSV, la-CRSV-5 MRC-38-25°C, MRC1-33°C Purified by MLD	VR-2566	2 April 1997 (02.04.97)
RSV, Line 19 MRC5 72-35°C VERO 3-35°C Large Clone 6-35°C, (3PI) Plaque Purified	VR-2572	23 April 1997 (23.04.97)
Progenitor wt RSV, line 19 MRC5 1-33°C, VERO 2-35°C, MRC5 1-35°C Purified by MLD	VR-2570	23 April 1997 (23.04.97)
Progenitor wt RSV, WI 38-3 MRC5 9-35°C, MRC5 1-35°C, Purified by MLD	VR-2571	23 April 1997 (23.04.97)

Applicant's or agent's file reference number	2115S01113POC	International application No. PCT/US 98/06636

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on pages 4 and 5, line ______

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Name of depositary institution

American Type Culture Collection

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
20 September 1995 (20.09.95)	VR-2511 (see additional sheet)

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet [ ]

In respect of the designation of the United Kingdom, a sample should be made available only to an expert.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

United Kingdom

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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Continuation Of Indications Relating To A Deposited Microorganism
(PCT Rule 13*bis*)
For United Kingdom

Identification Of Deposit	American Type Culture Collection Accession Number	Date of Deposit
RSV, Line 19 MRC5-15-25° st-33°	VR-2512	20 September 1995 (20.09.95)
RSV, Line 19 MRC5-60-35°	VR-2513	20 September 1995 (20.09.95)
RSV, Line 48 MRC5-14-25° st MRC1-33° VERO 10-25° VERO 1-33°	VR-2514	20 September 1995 (20.09.95)
RSV, Line 19 MRC5-10-25° VERO16-25° VERO 6-20° VERO 3-33°	VR-2515	20 September 1995 (20.09.95)
RSV, CRSV-BC5 CL-17 MRC30	VR-2516	20 September 1995 (20.09.95)
RSV, CRSV-BC13 MRC19-25° MRC1-33°	VR-2517	20 September 1995 (20.09.95)
RSV, Line 19 MRC5 92-35°C, Clone 4-1, MRC5, P-103-33° Purified by minimal limited dilution (MLD)	VR-2567	2 April 1997 (02.04.97)
RSV, Line 19 MRC 70-35°C, Vero 3-35°C, MRC5 2-35°C Clone 2-35°C (3PI) Plaque purified	VR-2564	2 April 1997 (02.04.97)
RSV, Line 19 MRC5, 92-35°C Clone 5-1 Purified by MLD	VR-2565	2 April 1997 (02.04.97)
RSV, la-CRSV-5 MRC-38-25°C, MRC1-33°C Purified by MLD	VR-2566	2 April 1997 (02.04.97)
RSV, Line 19 MRC5 72-35°C VERO 3-35°C Large Clone 6-35°C, (3PI) Plaque Purified	VR-2572	23 April 1997 (23.04.97)
Progenitor wt RSV, line 19 MRC5 1-33°C, VERO 2-35°C, MRC5 1-35°C Purified by MLD	VR-2570	23 April 1997 (23.04.97)
Progenitor wt RSV, WI 38-3 MRC5 9-35°C, MRC5 1-35°C, Purified by MLD	VR-2571	23 April 1997 (23.04.97)

Applicant's or agent's file reference number	2115S01113POC	International Application No. [illegible]

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on pages 4 and 5, line

B. IDENTIFICATION OF DEPOSIT RSV, 1a-CRSV-5 CL 15 MRC27 — Further deposits are identified on an additional sheet [X]

Name of depositary institution

American Type Culture Collection

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
20 September 1995 (20.09.95)	VR-2511 (see additional sheet)

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet [ ]

In respect of the designation of the EPO, samples of the deposited micro-organisms will be made available until the publication of the mention of the grant of the European patent or until the date on which the application is refused or withdrawn or is deemed to be withdrawn, as provided in Rule 28(3) of the Implementing Regulations under the EPC only by the issue of a sample to an expert nominated by requester (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

EPO

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
[ ] This sheet was received with the international application	[ ] This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Continuation Of Indications Relating To A Deposit d Microorganism
(PCT Rule 13*bis*)
For United Kingdom

Identification Of Deposit	American Type Culture Collection Accession Number	Date of Deposit
RSV, Line 19 MRC5-15-25° st-33°	VR-2512	20 September 1995 (20.09.95)
RSV, Line 19 MRC5-60-35°	VR-2513	20 September 1995 (20.09.95)
RSV, Line 48 MRC5-14-25° st MRC1-33° VERO 10-25° VERO 1-33°	VR-2514	20 September 1995 (20.09.95)
RSV, Line 19 MRC5-10-25° VERO16-25° VERO 6-20° VERO 3-33°	VR-2515	20 September 1995 (20.09.95)
RSV, CRSV-BC5 CL-17 MRC30	VR-2516	20 September 1995 (20.09.95)
RSV, CRSV-BC13 MRC19-25° MRC1-33°	VR-2517	20 September 1995 (20.09.95)
RSV, Line 19 MRC5 92-35°C, Clone 4-1, MRC5, P-103-33° Purified by minimal limited dilution (MLD)	VR-2567	2 April 1997 (02.04.97)
RSV, Line 19 MRC 70-35°C, Vero 3-35°C, MRC5 2-35°C Clone 2-35°C (3PI) Plaque purified	VR-2564	2 April 1997 (02.04.97)
RSV, Line 19 MRC5, 92-35°C Clone 5-1 Purified by MLD	VR-2565	2 April 1997 (02.04.97)
RSV, la-CRSV-5 MRC-38-25°C, MRC1-33°C Purified by MLD	VR-2566	2 April 1997 (02.04.97)
RSV, Line 19 MRC5 72-35°C VERO 3-35°C Large Clone 6-35°C, (3PI) Plaque Purified	VR-2572	23 April 1997 (23.04.97)
Progenitor wt RSV, line 19 MRC5 1-33°C, VERO 2-35°C, MRC5 1-35°C Purified by MLD	VR-2570	23 April 1997 (23.04.97)
Progenitor wt RSV, WI 38-3 MRC5 9-35°C, MRC5 1-35°C, Purified by MLD	VR-2571	23 April 1997 (23.04.97)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/06636

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/155
US CL : 424/89
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/89

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, MEDLINE, WPIDS, CAPLUS
search terms: Respiratory syncytial virus, RSV, parainfluenza, vaccine, attenuat?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RANDOLPH et al. Attenuated temperature-sensitive respiratory syncytial virus mutants generated by cold adaptation. Virus Research. 1994, Vol. 33, pages 241-259, see the entire document.	1-22
Y	CROWE, JR. et al. Satisfactorily attenuated and protective mutants derived from a partially attenuated cold-passaged respiratory syncytial virus mutant by introduction of additional attenuating mutations during chemical mutagenesis. Vaccine. 1994, Vol. 12, No. 8, pages 691-699, see the entire document.	1-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 MAY 1998	30 JUL 1998

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	ALI R. SALIMI
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/06636

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CROWE, JR. et al. A comparison in chimpanzees of the immunogenicity and efficacy of live attenuated respiratory syncytial virus (RSV) temperature-sensitive mutant vaccines and vaccinia virus recombinants that express the surface glycoproteins of RSV. Vaccine. 1993, Vol. 11, No. 14, pages 1395-1404, see the entire document.	1-22
A	MURPHY et al. An update on approaches to the development of respiratory syncytial virus (RSV) and parainfluenza virus type 3 (PIV3) vaccines. Virus Research. 1994, Vol. 32, pages 13-36.	1-22
A	WRIGHT et al. Administration of a Highly Attenuated, Live Respiratory Syncytial Virus Vaccine to Adults and Children. Infection and Immunity. July 1982, Vol. 37, No. 1, pages 397-400.	1-22
A	MAASSAB et al. Development and characterization of cold-adapted viruses for use as live vaccines. Vaccine. December 1985, Vol. 3, No. 4, pages 355-369.	1-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/06636

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claim(s) 1-4 and 7, drawn to specific attenuated respiratory syncytial virus (RSV), and the first use (vaccine). (first product, and the first use).

Group II, claim(s) 5 and 6, drawn to method of determining the presence of antibodies against the RSV. (second use of the first product).

Group III, claim(s) 8-10, drawn to a composition comprising attenuated RSV, and a vaccine. (second product, and the use of the product).

Group IV, claim(s) 11 and 12, drawn to various mutants of RSV. (third product).

Group V, claim(s) 13-17 and 20-22, drawn to RSV selected from group wt19, and WRSV, and a vaccine. (forth product, and the first use of the product).

Group VI, claim(s) 18-19, drawn to method of determining antibodies of the forth product.(second use of the forth product).

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features (S.T.F) for the following reasons: The specific technical feature of Group I is the characteristics of the specific attenuated viruses listed in claim 1. Second product does not require special technical features of Group I because it is broader in scope than Group I and does not require the particular viruses required by Group 1. The third product, election of species is also required for explanation see below, does not require special technical features of Group I, because each virus in Group I has multiple mutations contributing to the characteristics of each strain. Since the special technical features of group I involves the combined effect of multiple mutations, the single mutations in isolation do not share the same special technical features. The forth product does not require the special technical features of Group I because it is drawn to different viruses.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: Explanation of election of species in the third product Group IV: Each mutation listed confers different structure and presumably different effect on antigenicity and virulence of the virus. Since effects of mutations are unpredictable, a mutation of one location does not teach or suggest mutation at a different location. Therefore, each location is a distinct species.